Differential Cortical Aspartate Uptake Across the Oestrous Cycle is Associated with Changes in Gut Microbiota in Wistar-Kyoto Rats

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Abstract

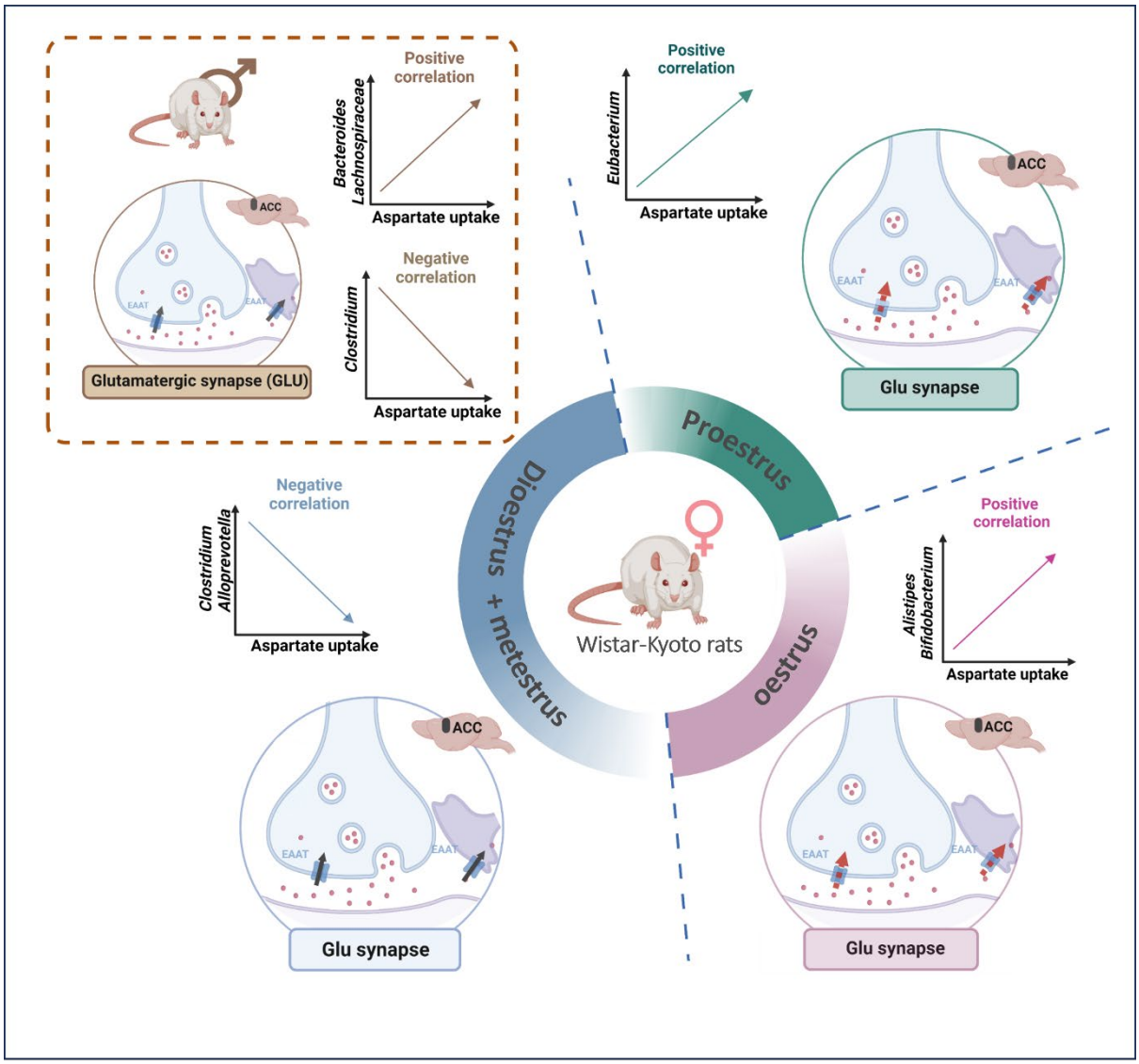
Pain and psychological stress are intricately linked, with sex differences evident in disorders associated with both systems. Glutamatergic signalling in the central nervous system is influenced by gonadal hormones via the hypothalamic-pituitary-adrenal axis and is central in pain research. Emerging evidence supports an important role for the gut microbiota in influencing pain signalling. Here, the functional activity of excitatory amino acid transporters (EAATs) in the anterior cingulate cortex (ACC) and lumbosacral spinal cord of male and female Wistar-Kyoto rats, an animal model of comorbid visceral hypersensitivity and enhanced stress responsivity, was investigated across the oestrous cycle. Correlations between the gut microbiota and changes in the functional activity of the central glutamatergic system were also investigated.

EAAT function in the lumbosacral spinal cord was similar between males and females across the oestrous cycle. EAAT function was higher in the ACC of dioestrus females compared to proestrus and oestrus females. In males, aspartate uptake in the ACC positively correlated with *Bacteroides*, while aspartate uptake in the spinal cord positively correlated with the relative abundance of *Lachnospiraceae NK4A136*. Positive associations with aspartate uptake in the spinal cord were also observed for *Alistipes* and *Bifidobacterium* during oestrus, and *Eubacterium coprostanoligenes* during proestrus. *Clostridium sensu stricto1* was negatively associated with aspartate uptake in the ACC in males and dioestrus females.

These data indicate that glutamate metabolism in the ACC is oestrous stage-dependent and that short-chain fatty acid-producing bacteria are positively correlated with aspartate uptake in males and during specific oestrous stages in females.

Significance statement

The data from this manuscript indicates that glutamate metabolism in the anterior cingulate cortex is oestrous stage-dependent and that short-chain fatty acid-producing bacteria are positively correlated with aspartate uptake in males and during specific oestrous stages in females.



Introduction

Chronic stress has been linked to several disorders including functional pain syndromes, autoimmune disorders, and depression [1-3]. In fact, patients with chronic functional pain syndromes such as irritable bowel syndrome (IBS) exhibit altered baseline plasma levels of noradrenaline, supporting a potential relationship between stress and pain disorders [4, 5]. Repeated or prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis by internal or external stressors has been implicated in the development of both depression and IBS [6-8] and can result in nervous system dysregulation such as central sensitisation [9] and the development of chronic pain states [10-12].

Visceral pain can be influenced by the gut microbiota [13, 14]. Probiotics, beneficial bacteria, have been shown to decrease stress or antibiotic-induced visceral hypersensitivity in rodent models [15-17]. In clinical studies, microbiota-targeted interventions have also been shown to alleviate abdominal pain [18]. Recently, distinct sex-dependent clusters in the gut microbiota have been found to precede behavioural phenotypes related to visceral pain in adulthood [19]. Interestingly, positive correlations have also been observed between pain sensation thresholds and specific stages of the menstrual cycle and bacterial genera [14]. Intestinal microbial richness and functions have been shown to modulate the oestrous cycle in rats [20] and oestrogen levels in humans [21] and the gut microbiome was found to modulate variations in visceral pain across the oestrous cycle [22].

The Wistar-Kyoto (WKY) rat strain demonstrates hyperresponsivity to stress [23, 24] and elevated basal and stress-state corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and corticosterone levels compared to other strains [25, 26]. These animals have decreased expression levels of glial fibrillary acidic protein (GFAP), an astrocytic marker [27], and increased neuronal activation in the rostral anterior cingulate cortex (ACC), a key

94 brain region involved in the affective components of pain [28-30] in response to pain
95 compared to Sprague-Dawley (SD) rats [31]. Changes in glial activation have been shown to
96 modify glial glutamate metabolism through direct effects on the activity of excitatory amino
97 acid transporters (EAATs), a family of transmembrane transporters mainly found in astrocytes
98 and neurons, [32, 33] which has also been implicated in major depression [34]. Previously,
99 male and female WKY rats have been shown to be more sensitive to painful stimuli compared
100 to SD rats of the same sex [35, 36]. Female WKY rats presented lower pain thresholds and
101 increased nociceptive behaviours compared to male WKY rats [37], however, responses to
102 painful stimuli across the oestrous cycle in this strain have yet to be fully explored.
103 The glutamatergic system is known to play a key role in the initiation, processing, and
104 maintenance of pain [38]. Glutamate is released from primary afferent neurons into the
105 dorsal horn of the spinal cord and contributes to the diffusion of pain signals. Intrathecal
106 injections of a glutamate receptor antagonist at the level of the lumbar spinal cord have been
107 shown to block stress-induced hyperalgesia in rats [39]. Glutamate is also the major fast
108 excitatory neurotransmitter in the central nervous system (CNS) and is present in the ACC.
109 Overactivation of the glutamatergic system in the ACC has been linked to chronic pain states
110 [40, 41] and to an increased nociceptive behaviour in female rats [42]. Interestingly, EAATs,
111 seem to play a crucial role in glutamate homeostasis through rapid glutamate uptake from
112 the synaptic cleft [43]. Variations in visceral sensitivity and aberrant spinal and ACC excitatory
113 amino acid transport were observed in response to stress and gonadal hormone fluctuations
114 across the oestrous cycle in female rats [44].
115 There is growing evidence to support the role of gonadal hormones in the regulation of the
116 HPA axis with, for instance, differences in cortisol response being noted between male and
117 female patients suffering from IBS [45] and depression [46, 47]. In rodents, adult females

exhibit higher stress-state levels of ACTH and corticosterone compared to males following both acute and repeated restraint [48]. Similarly, sex differences in pain-related aversive behaviours were observed following optogenetic activation or inhibition of glutamatergic receptors in the ACC [42]. In female rodents, the excitability of CRH neurons was found to fluctuate across the oestrous cycle with higher levels of excitability during high-oestrogen stages, i.e. proestrus, when compared to other stages [49]. Fluctuations in the circulating levels of oestrogen during the menstrual cycle can also lead to variations in the symptomatology of chronic pain syndromes through the activation of glutamatergic receptors [50-52]. In female rats, pain-related aversive behaviours were attenuated through inhibition of oestrogen receptors in the ACC [52]. In the spinal cord, oestrogen has also been shown to modulate spinal glutamatergic homeostasis [53-55]. Female rats in high-oestrogen states exhibit reduced EAAT function in the ACC and spinal cord compared to low-oestrogen states [44]. Additionally, gonadal hormones significantly affect visceral sensitivity in animal models, with oestrogen demonstrating pronociceptive effects in rats [53-56].

However, there is a paucity of literature examining the interaction between the oestrous cycle and glutamate signalling in key pain-processing regions of the CNS and whether it is related to the gut microbiota. Thus, given this evidence we hypothesised that glial aspartate uptake in the spinal cord and/or ACC may follow the natural gonadal hormone fluctuations over the oestrous cycle in female WKY rats in comparison to males. We also explored if correlations were evident between changes in functional activity of the EAATs and the caecal microbiota, and whether these were dependent on gonadal hormones.

Material and Methods

Animals

Thirty adult females (125-150g) and ten males (150-175g) were purchased from Envigo, UK and were group housed (4-5 animals per sex per cage) in plastic cages in a humidity- and temperature- (21°C ± 1°C) controlled room in the Biological Services Unit of University College Cork. Food and water were available ad libitum, and the light/dark cycle was set to 12 hours (lights on at 7:00am). Rats were allowed to habituate in the new environment for 1 week before the commencement of experiments.

A number of samples were not suitable for analysis due to damage upon collection: 1 female (in proestrus) for spinal cord analysis and 3 females (2 in proestrus and 1 in oestrus) for ACC analysis. All experiments were in full accordance with the European Community Council Directive (2010/63/EU), and approved by the Animal Experimentation Ethics Committee of University College Cork (2012/045) and the Health Products Regulatory Authority. All animals were visually checked daily and all efforts were made to reduce stress in the holding room. All cages were changed once weekly, and environmental enrichment was provided.

Vaginal Smears

Female rats were vaginally lavaged [57] immediately before euthanasia, to accurately determine oestrous stage for all collected tissue. Samples were collected via vaginal lavage with saline, and cells were analysed under a microscope. The stage of the oestrous cycle was determined as previously described [58]. As metestrus only lasts for a short period (5-6 hours), and the plasma oestrogen concentration in metestrus is similar to that during dioestrus, these two stages were combined and assessed together [22, 44].

Male rats were handled for the same duration as females, but no additional procedures were performed.

Sample Preparation for Aspartate Transport Assay

Animals were euthanised by decapitation and spinal cords were removed by hydraulic pressure into Hanks' balanced salt solution (HBSS)-filled Petri dishes. Slices (400μm) were obtained from the lumbosacral spinal cord using a McIlwain tissue chopper. Brain tissue was removed from the skull and coronally sectioned using a vibratome to obtain 400μm-thick ACC sections. These slices were separated by fine dissection under a microscope and transferred to 24-well culture plates filled with either sodium-containing HBSS at 35°C, or sodium-free HBSS kept on ice. Slices from the sodium-enriched plate were washed once with 1mL of 35°C HBSS, and slices from the sodium-free plate were washed with 1mL of 4°C sodium-free HBSS to assess sodium-dependent and independent uptake, respectively.

Aspartate Transport Assay

Since EAATs show high affinity for both glutamate and aspartate, aspartate was used. This protocol has been previously described for brain and spinal cord slices [59, 60]. Aspartic Acid, D-[2,3-^{3}H] (specific activity 12.9Ci/mmol) was purchased from Perkin-Elmer, USA. Radioimmunoprecipitation assay (RIPA) buffer and Pierce bicinchoninic acid (BCA) protein assay kit was purchased from Fisher Scientific Ireland. All other reagents were purchased from Sigma-Aldrich. HBSS was prepared containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, balanced to pH 7.2. In sodium-free HBSS, NaCl was replaced by 137mM N-methyl-D-glucamine.

Spinal cord and ACC slices were pre-incubated at 35°C and 4°C in sodium-enriched and sodium-free HBSS, respectively, for 30 minutes. Following this, a solution containing 1µL of 0.66µCi/mL aspartic acid D-[2,3-^{3}H], 20µL of cold 100µM D-Aspartate and 84µL of H_2O was added. After 3 minutes of incubation for the spinal cords and 7 minutes for the ACC, the slices were washed twice with 1ml of corresponding ice-cold HBSS. Tissues were transferred into 1.5mL tubes containing RIPA buffer and were mechanically dissociated with pestles. This mixture was homogenised for 15 minutes at 4°C, and the residue was removed. Radioactivity was measured in terms of counts per minute (CPM) using a liquid scintillation counter and the results for sodium-free samples were subtracted from those of sodium-enriched samples to achieve sodium-dependent uptake-hallmark of EAAT 1 and 2 function. The uptake procedure was performed in triplicate for each rodent. Protein levels were quantified using Pierce BCA protein assay kit. The final scintillation result for each slice was divided by respective protein quantity to achieve aspartate uptake in terms of CPM/mg, which is indicative of spinal and cortical EAAT function.

Caecal Microbiota Analysis

DNA Extraction and PCR

DNA was extracted from the contents of the caecum, sequenced by Illumina MiSeq, and bioinformatics analyses were performed. Briefly, a 0.25g aliquot of sample was added to 1mL lysis buffer (500mM NaCl, 50mM Tris-HCl, pH 8.0, 50mM EDTA, and 4% sodium dodecyl sulfate) together with 0.4g of zirconia beads. The samples were homogenised for 3 minutes using the Biospec Mini-beadbeater at maximum speed. The homogenised samples were then heated to 70°C for 15 minutes and then centrifuged at 16,000g for 5 minutes. The supernatant was then removed, fresh lysis buffer added, and the beadbeating, heating, and centrifugation steps were repeated. The nucleic acids were then precipitated using 10M ammonium acetate,

followed by the addition of isopropanol. The nucleic acids were then pelleted, washed, and resuspended in 1X TE buffer. Subsequently, removal of RNA, protein, and purification of the DNA was completed using components of the QIAGEN QIAamp DNA Stool Mini kit along with the wash buffers and elution buffer, and DNA was stored at -20°C.

The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina), as is standard practice for microbiome analysis [61]. The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene, which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each PCR reaction contained DNA templates, 5µL of forward primers (1µM), 5µL of reverse primers (1µM), 12.5µL of 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), and PCR-grade water to a final volume of 25µL. PCR amplification was carried out as follows: heated lid 110°C, 95°C x 3 minutes, 25 cycles of 95°C x 30 s, 55°C x 30 s, 72°C x 30 s, then 72°C x 5 minutes and held at 4°C. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose, 100V). Successful PCR products were cleaned using AMPure XP magnetic bead-based purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA (5µl) to index each of the samples, allowing samples to be pooled for sequencing on the one flow cell and subsequently demultiplexed for analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5µL index 1 primer (N7xx), 5µL index 2 primer (S5xx), 25µL 2x Kapa HiFi Hot Start Ready mix, and 10µL PCR grade water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 25. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified

using the Qubit™ 3.0 Fluorometer (Biosciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit. Samples were then pooled at equimolar concentrations. The sample pool was prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 x 250 cycle kit, following standard Illumina sequencing protocols.

Bioinformatic Analysis

250 base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME Version 1.9.0. Singleton removal (to ensure a minimum of 2 copies to be counted), denoising, chimera detection, and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123. Data were not rarefied/normalised for further analysis. The richness and α-diversity indices were generated in QIIME Version 1.9.0 [62].

Statistical Analysis

Data are expressed as mean ± SEM. The threshold of statistical significance was set at $p<0.05$. Spearman rank correlation coefficients were calculated for determination of the existence of significant associations between aspartate uptake and microbial relative abundances in the gut. P-values were corrected for multiple comparisons as described by Benjamini and

264 Hochberg [63]. Finally, a Canonical Correspondence Analysis (CCA) was carried out using the

265 vegan package in R [64].

Results

Sex and oestrous cycle have no effect on spinal glial aspartate uptake

An independent samples t-test found no significant difference in aspartate uptake in the lumbosacral spinal cord between male and female WKY rats ($t(35) = 1.463$, $p = 0.152$; Figure 1A). To investigate the potential impact of the oestrous cycle on aspartate uptake in the lumbosacral spinal cord, a one-way ANOVA performed between the various oestrous stages revealed no significant difference between the groups ($F(2,28) = 0.34$, $p = 0.966$; Figure 1B).

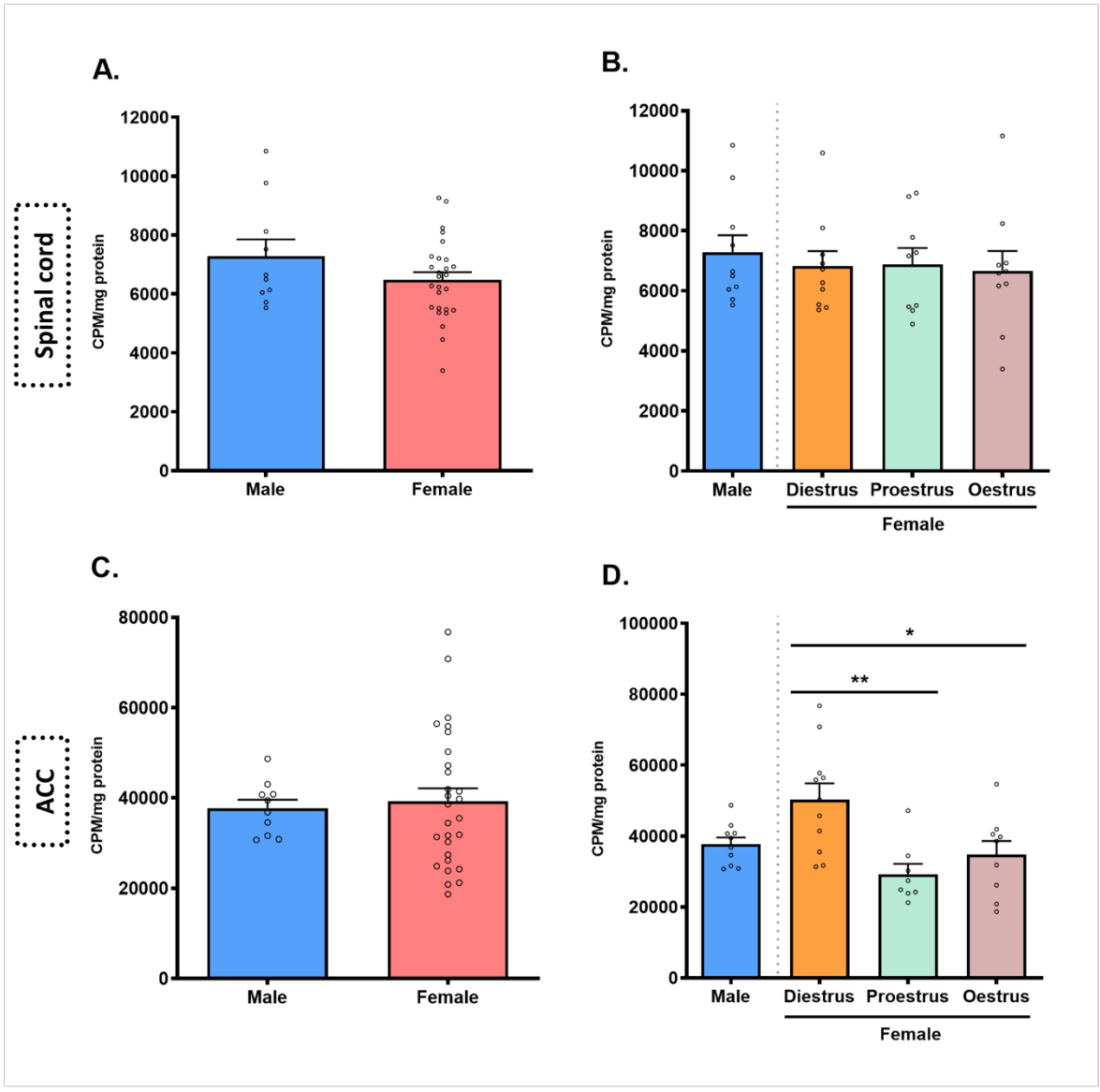


Figure 1. Aspartate uptake is reduced in the high-oestrogen stages of the oestrous cycle in the anterior cingulate cortex (ACC). Aspartate uptake was assessed as a function of sex and the oestrous cycle in the

lumbosacral spinal cord (A and B) and anterior cingulate cortex (C and D). **(A)** In the spinal cord, aspartate uptake was similar in between males (n = 10) and all females (n = 27) **(B)** females across all oestrous stages (n=9-10/group). **(C)** In the ACC, aspartate uptake in males (n = 10) and all females (n = 28) was similar **(D)** significant oestrogen-dependent changes in females (n = 8-11/group). Uptake was higher in dioestrus compared to proestrus females. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

Aspartate Uptake in ACC relates to oestrous Cycle stage

To compare the uptake of aspartate in the ACC between male and female WKY rats, a Welsh's t-test was performed, revealing no significant difference ($t(35.531) = -0.458$, $p = 0.65$; Figure 1C). To assess the potential impact of the oestrous cycle on ACC aspartate uptake, a one-way ANOVA between the 3 female groups revealed a significant difference in aspartate uptake between the groups ($F(2,27) = 7.681$, $p = 0.003$; Figure 1D). Further analysis using Tukey's HSD post hoc test revealed that ACC aspartate uptake was higher in dioestrus compared to proestrus ($p = 0.003$) and versus oestrus ($p = 0.025$) (Figure 1D).

Associations between bacterial relative abundances in the gut and aspartate uptake in the ACC and spinal cord

Changes in relative abundances of the most prominent bacterial taxa are illustrated as a function of increasing levels of aspartate uptake in the ACC and spinal cord between samples at the genus level in females as well as in males (Figure 2A). CCA revealed that the effect of aspartate uptake in the ACC on bacterial dispersion was orthogonal to the effect exerted by aspartate uptake in the spinal cord (Figure 2B). However, neither aspartate uptake in the ACC ($p = 0.912$) nor in the spinal cord ($p = 0.586$) were found to significantly influence the gradient of bacterial dispersion between samples with a clear majority of taxa observed to cluster together at the centre of the plot. Sample groups also failed to form distinct clusters indicating

303 that differences in the relationships between bacterial abundances of the gut microbiota and
304 aspartate uptake in the ACC and spinal cord are not influenced by sex or oestrous cycle stage.
305 Despite this, significant positive associations were observed in the male rats between
306 aspartate uptake in the ACC and the *Bacteroides* (q = 0.0003; Spearman's rho 0.967).
307 *Alloprevotella* correlated negatively (q = 0.0145; Spearman's rho -0.881) with ACC aspartate
308 uptake during the dioestrus stage. *Clostridium sensu stricto 1* negatively correlated with
309 aspartate uptake in dioestrus females (q = 0.0091; Spearman's rho -0.905) and males
310 (q=0.0215; Spearman's rho -0.817). Aspartate uptake in the spinal cord was found to correlate
311 positively with relative abundances of the *Lachnospiraceae NK4A136 group* (q = 0.0427;
312 Spearman's rho 0.766) in males. Further positive associations with aspartate uptake in the
313 spinal cord were also observed for *Alistipes* (q = 0.0344; Spearman's rho 0.783) and
314 *Bifidobacterium* (q = 0.0427; Spearman's rho 0.766) during oestrus in females, as well as for
315 the *Eubacterium coprostanoligenes group* (q = 0.0333; Spearman's rho 0.943) during
316 proestrus (Figure 2C).

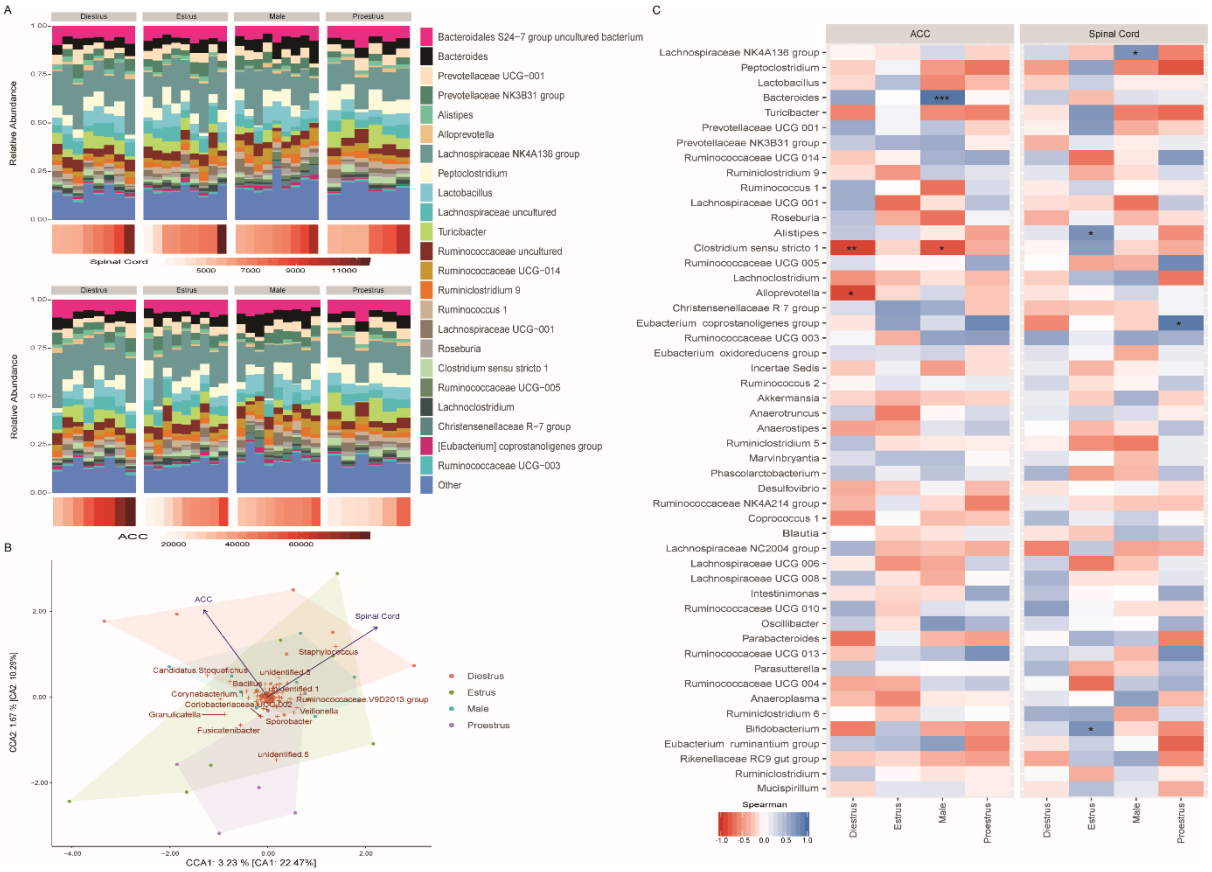


Figure 2. Associations between bacterial relative abundances and aspartate uptake in the ACC and spinal cord. **A)** The influence of increasing aspartate uptake, in the ACC and spinal cord respectively, on sample relative abundances at the genus level, both in males and across the oestrous cycles in females. **B)** CCA of microbiota composition regressed on measures of aspartate uptake in both the ACC and spinal cord. Filled circles represent samples while red crosses represent bacterial taxa (genus level) with the top 5% of taxa which best fit the axes being indicated by name. The ACC and spinal cord vectors both point to the direction of taxa which exhibit the strongest association with the variable while their magnitude indicate the strength of the variable in explaining the bacterial dispersion observed. The canonical variates explain 14.4% and 16.3% of the total explainable variance in the first and second axis respectively. **C)** Heat map of the 50 most abundant bacterial taxa (genus level) correlated against aspartate uptake in the ACC and spinal cord using Spearman's correlation (* $q < 0.05$; ** $q < 0.01$; *** $q < 0.001$; q = False discovery rate adjusted p-value (q-value) after Benjamini-Hochberg multiple testing corrections).

Discussion

Our results reveal a correlation between oestrous stage and the function of sodium-dependent glutamate transporters in female WKY rats. Cortical EAAT activity was reduced in the proestrus and oestrus stages when compared to the dioestrus stage of the oestrous cycle. However, there were no changes in glutamate metabolism at the lumbosacral spinal cord level. In males, aspartate uptake in the ACC and spinal cord was found to correlate positively with *Bacteroides* and *Lachnospiraceae NK4A136,* respectively. In the spinal cord, *Alloprevotella* was negatively correlated with aspartate uptake in dioestrus females, and *Clostridium sensu stricto 1* negatively correlated with aspartate uptake in males and dioestrus females. In females, positive associations with aspartate uptake in the spinal cord were observed for *Alistipes* and *Bifidobacterium* during oestrus, and for the *Eubacterium coprostanoligenes* during proestrus.

The lumbosacral spinal cord is an important part of the CNS in relation to the processing of pain signals from most of the abdominal viscera. Afferents from the viscera play only a small role in the hormonal modulation of pain responses [65], however a decreased expression of EAAT in the spinal cord of female WKY rats exposed to chronic stress has been associated with increased pain responses [36]. In our study, there was no change observed in the function of lumbosacral spinal EAATs between the sexes or across the oestrous cycle. This may suggest that in this animal strain, the hormonal state-dependent function of EAATs may play a larger role in pain processing along the higher ascending pain pathways rather than the lumbosacral spinal cord.

The ACC interconnects with other key brain regions involved in cognition and behaviour such as the frontal cortex, the thalamus, and the amygdala [66, 67]. Clinical studies have reported activation of the ACC using imaging techniques in response to painful stimuli, supporting its

role in pain processing [68]. The rostral area of the ACC is thought to be particularly involved in the processing of the affective component of pain [69] and an increased activation of glutamate receptors in the ACC has been observed in viscerally-hypersensitive rats [70].

As chronic pain and depression are closely related, various studies have investigated and elucidated the role of the ACC in the development of depression [71]. Reductions in ACC volume visualised using MRI have been reported in patients with depression [72], and some studies have shown ablative procedures such as anterior cingulotomy to be effective in treatment-resistant depression [73]. A reduction in the volume of the ACC in depressed patients is believed to be caused by a decrease in glial density rather than neuronal tissue [74, 75]. Since glial cells participate in the uptake and recycling of glutamate, a reduction in glial density may be accountable for the reported decrease in glutamate levels in patients with depression [76, 77]. In WKY rats, which are also an animal model of depression-like behaviour [23], glutamate receptors have been reported as either less functional or less numerous in female hippocampi and frontal cortices [78].

The gut microbiota can significantly influence the structure and functioning of the ACC. In germ-free (GF) mice, the volume of the ACC was seen to be reduced along with dendritic changes [79], whilst ovariectomy-induced visceral pain was not evident in GF mice [22]. Our study indicated that *Bacteroides* had a sex-specific positive correlation with ACC glial aspartate uptake in male rats. While the findings of this study are correlative and causality may not be implied, some *Bacteroides* species have been previously shown to modulate depression-like behaviour through gut-brain signalling pathways [80, 81]. Further, *Lachnospiraceae* and *Alistipes* both displayed positive correlations with lumbar spinal glial aspartate uptake in males and oestrus female rats, respectively. *Lachnospiraceae* has been found to be reduced in patients with IBS displaying comorbid depression/anxiety [82] and in

stress models exhibiting depression-like behaviours [83]. In other rodent models, the probiotic *Bifidobacterium longum* has been shown to reduce symptoms of depression [84]. In our study, *Bifidobacterium* correlated positively with spinal aspartate uptake in oestrus female rats. Further, short-chain fatty-acid (SCFA)-producing bacteria, including *Bacteroides*, *Lachnospiraceae*, and *Bifidobacterium* had a positive correlation with aspartate uptake in the ACC and spinal cord in a sex-dependent manner. Different SCFA's elicit opposite effects on pain sensitivity. For instance, butyrate has been shown to alleviate visceral pain [85], while the role of acetate and propionate are less studied but both are found in lower levels in patients with irritable bowel disease and fibromyalgia, respectively [86]. This presents a unique opportunity to potentially alter visceral sensitivity via modulation of SCFA composition by administration of probiotics.

Oestrogen has a significant influence on glutamate metabolism through the regulation of EAATs via the nitric oxide pathway [87]. It has been previously shown that oestradiol triggers the production of nitric oxide by coupling NMDA receptors (EAATs) to neuronal nitric oxide synthase, thus activating neuronal nitric oxide signalling [88]. This may represent an avenue of further study to better understand pain disorders in females given the cyclic changes in oestrogen across the menstrual cycle. In our study, in the high-oestrogen state, i.e. proestrus and ensuing oestrus, a markedly decreased activity of EAATs in the ACC compared to dioestrus was observed. A similar link between oestrous cycle and EAAT activity had been previously reported and also associated with increased visceral pain in Sprague-Dawley females [44]. This supports an oestrous cycle-dependent modulation of glutamate metabolism in the ACC, possibly leading to a heightened pain sensitivity. SCFAs have been shown to enhance cellular oestrogen sensitivity through the mitogen-activated protein kinase pathway [89]. Through this pathway, SCFA-producing gut bacteria may have shown positive

correlations with aspartate uptake in WKY rats that exhibit phenotypic features of depression with altered ACC structure and function.

Finally, our data suggest that while there is no impact of the oestrous cycle on the glutamate transporters in the lumbosacral spinal cord, excitatory glial transport in the ACC is dependent on the oestrous cycle stage of the WKY female rats and the gut microbiota may be an important modulating factor. These data agree with previous findings linking variations in pain threshold and gut microbiome across the menstrual cycle [14]. As the ACC is related to the affective aspect of pain, our study supports the need for future preclinical and clinical trials evaluating pain-associated behavioural traits in both sexes and across the oestrous/menstrual cycle, with an emphasis on the role of the gut microbiota. By identifying microbial strains or communities that play a role in the apparition of visceral pain, such targeted approaches may inform the next generation of microbiota-targeted interventions for the management of pain.

Overall, the data from this manuscript suggests that glutamate metabolism in the ACC is oestrous stage-dependent and that SCFA-producing bacteria are positively correlated with aspartate uptake in males and during specific oestrous stages in females.

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Conflict of interest

All authors acknowledge no conflict of interest. SMO'M has received funding from Mead Johnson. JFC has spoken at conferences organised by Mead Johnson, Ordesa, and Yakult and has received research funding from Reckitt, Nutricia, Dupont/IFF, and Nestle. GC has received honoraria from Janssen, Probi, Apsen and Boehringer Ingelheim as an invited speaker; has received research funding from Pharmavite, Fonterra, Tate and Lyle, Reckitt and Nestle; and has been a consultant/advisor for Yakult, Zentiva, Heel Pharmaceuticals and Bayer Consumer Healthcare. This support neither influenced nor constrained the contents of this manuscript.

Data availability statement

All data reported in this paper will be shared by the corresponding author upon reasonable request.

Author contributions

J. Saijad: conceptualisation, data curation, formal analysis, investigation, visualisation, writing – original draft, writing – review & editing; J. Morael: writing – original draft, writing – review & editing; T.G. Melo: writing – original draft, writing – review & editing; T. Foley: data curation, investigation, methodology, resources; A. Murphy: formal analysis; J. Keane: formal analysis; J. Popov: writing – original draft; C. Stanton: data curation, methodology, resources; T.G. Dinan: funding acquisition, conceptualisation, supervision, writing – original draft; G. Clarke: writing – original draft, writing – review & editing: J.F. Cryan: funding acquisition, writing – original draft, writing – review & editing, supervision; J.M. Collins: formal analysis, visualisation, writing – original draft, writing – review & editing; S.M.O'M: conceptualisation, data curation, funding acquisition, investigation, methodology, supervision, writing – original draft, writing – review & editing.

Reference:

1. H Yaribeygi, Y Panahi, H Sahraei, et al., *The impact of stress on body function: A review.* EXCLI Journal, 2017. 16: p. 1057-1072.
2. L Stojanovich and D Marisavljevich, *Stress as a trigger of autoimmune disease.* Autoimmunity Reviews, 2008. 7(3): p. 209-213.
3. L J Crofford, *Chronic Pain: Where the Body Meets the Brain.* Transactions of the American Clinical and Climatological Association, 2015. 126: p. 167-183.
4. R D Moloney, A C Johnson, S M O'Mahony, et al., *Stress and the Microbiota-Gut-Brain Axis in Visceral Pain: Relevance t o Irritable Bowel Syndrome.* CNS neuroscience & therapeutics, 2016. 22(2): p. 102-117.
5. M Gros, B Gros, J E Mesonero, et al., *Neurotransmitter Dysfunction in Irritable Bowel Syndrome: Emerging App roaches for Management.* Journal of Clinical Medicine, 2021. 10(15): p. 3429.
6. C Park, J D Rosenblat, E Brietzke, et al., *Stress, epigenetics and depression: A systematic review.* Neuroscience and Biobehavioral Reviews, 2019. 102: p. 139-152.
7. E A Mayer, K Nance and S Chen, *The Gut-Brain Axis.* Annual Review of Medicine, 2022. 73: p. 439-453.
8. L Wilmes, J M Collins, K J O'Riordan, et al., *Of bowels, brain and behavior: A role for the gut microbiota in psychi atric comorbidities in irritable bowel syndrome.* Neurogastroenterology & Motility, 2021. 33(3): p. e14095.
9. O C Eller-Smith, A L Nicol and J A Christianson, *Potential Mechanisms Underlying Centralized Pain and Emerging Therapeu tic Interventions.* Frontiers in Cellular Neuroscience, 2018. 12: p. 35.
10. A Woda, P Picard and F Dutheil, *Dysfunctional stress responses in chronic pain.* Psychoneuroendocrinology, 2016. 71: p. 127-135.
11. M Beverley Greenwood-Van, lt, sup, et al., *Mechanisms of Stress-induced Visceral Pain.* Journal of Neurogastroenterology and Motility, 2018. 24(1): p. 7-18.
12. I Timmers, C W E M Quaedflieg, C Hsu, et al., *The interaction between stress and chronic pain through the lens of th reat learning.* Neuroscience and Biobehavioral Reviews, 2019. 107: p. 641-655.
13. S M O' Mahony, T G Dinan and J F Cryan, *The gut microbiota as a key regulator of visceral pain.* Pain, 2017. 158 Suppl 1: p. S19-S28.
14. V Caputi, T F S Bastiaanssen, V Peterson, et al., *Sex, pain, and the microbiome: The relationship between baseline gut m icrobiota composition, gender and somatic pain in healthy individuals.* Brain, Behavior, and Immunity, 2022. 104: p. 191-204.
15. E F Verdú, P Bercik, M Verma-Gandhu, et al., *Specific probiotic therapy attenuates antibiotic induced visceral hype rsensitivity in mice.* Gut, 2006. 55(2): p. 182-190.
16. D P McKernan, P Fitzgerald, T G Dinan, et al., *The probiotic Bifidobacterium infantis 35624 displays visceral antinoc iceptive effects in the rat.* Neurogastroenterology and Motility: The Official Journal of the Europe an Gastrointestinal Motility Society, 2010. 22(9): p. 1029-1035, e268.
17. K-A McVey Neufeld, C R Strain, M M Pusceddu, et al., *Lactobacillus rhamnosus GG soluble mediators ameliorate early life str ess-induced visceral hypersensitivity and changes in spinal cord gene expression.* Neuronal Signaling, 2020. 4(4): p. NS20200007.

18. A N Galica, R Galica and D L Dumitrașcu, *Diet, fibers, and probiotics for irritable bowel syndrome.* Journal of Medicine and Life, 2022. 15(2): p. 174-179.
19. L Wilmes, V Caputi, T F S Bastiaanssen, et al., *Sex specific gut-microbiota signatures of resilient and comorbid gut-brain phenotypes induced by early life stress.* Neurobiology of Stress, 2024. 33: p. 100686.
20. Y Guo, Y Qi, X Yang, et al., *Association between Polycystic Ovary Syndrome and Gut Microbiota.* PLOS ONE, 2016. 11(4): p. e0153196.
21. R Flores, J Shi, B Fuhrman, et al., *Fecal microbial determinants of fecal and systemic estrogens and estro gen metabolites: a cross-sectional study.* Journal of Translational Medicine, 2012. 10: p. 253.
22. M Tramullas, J M Collins, P Fitzgerald, et al., *Estrous cycle and ovariectomy-induced changes in visceral pain are mic robiota-dependent.* iScience, 2021. 24(8): p. 102850.
23. W P Paré, *Passive-avoidance behavior in Wistar-Kyoto (WKY), Wistar, and Fischer- 344 rats.* Physiology & Behavior, 1993. 54(5): p. 845-852.
24. S A Bassett, W Young, K Fraser, et al., *Metabolome and microbiome profiling of a stress-sensitive rat model of gut-brain axis dysfunction.* Scientific Reports, 2019. 9(1): p. 14026.
25. P A Rittenhouse, C López-Rubalcava, G D Stanwood, et al., *Amplified behavioral and endocrine responses to forced swim stress in the Wistar-Kyoto rat.* Psychoneuroendocrinology, 2002. 27(3): p. 303-318.
26. J C Lemos, G Zhang, T Walsh, et al., *Stress-Hyperresponsive WKY Rats Demonstrate Depressed Dorsal Raphe Neu ronal Excitability and Dysregulated CRF-Mediated Responses.* Neuropsychopharmacology, 2011. 36(4): p. 721-734.
27. R D Gosselin, S Gibney, D O'Malley, et al., *Region specific decrease in glial fibrillary acidic protein immunoreac tivity in the brain of a rat model of depression.* Neuroscience, 2009. 159(2): p. 915-925.
28. P N Fuchs, Y B Peng, J A Boyette-Davis, et al., *The anterior cingulate cortex and pain processing.* Frontiers in Integrative Neuroscience, 2014. 8: p. 35.
29. M D Lieberman and N I Eisenberger, *The dorsal anterior cingulate cortex is selective for pain: Results fr om large-scale reverse inference.* Proceedings of the National Academy of Sciences of the United States o f America, 2015. 112(49): p. 15250-15255.
30. V Pereira and C Goudet, *Emerging Trends in Pain Modulation by Metabotropic Glutamate Receptors.* Frontiers in Molecular Neuroscience, 2018. 11: p. 464.
31. S M Gibney, R D Gosselin, T G Dinan, et al., *Colorectal distension-induced prefrontal cortex activation in the Wist ar-Kyoto rat: implications for irritable bowel syndrome.* Neuroscience, 2010. 165(3): p. 675-683.
32. S M Sullivan, A Lee, S T Björkman, et al., *Cytoskeletal anchoring of GLAST determines susceptibility to brain dam age: an identified role for GFAP.* The Journal of Biological Chemistry, 2007. 282(40): p. 29414-29423.
33. R-R Ji, T Berta and M Nedergaard, *Glia and pain: Is chronic pain a gliopathy?* Pain, 2013. 154(0 1): p. S10-S28.
34. M N Fullana, A Covelo, A Bortolozzi, et al., *In vivo knockdown of astroglial glutamate transporters GLT-1 and GLAST increases excitatory neurotransmission in mouse infralimbic cortex: R elevance for depressive-like phenotypes.* European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology, 2019. 29(11): p. 1288-1294.

35. S M O' Mahony, G Clarke, D P McKernan, et al., *Differential visceral nociceptive, behavioural and neurochemical respo nses to an immune challenge in the stress-sensitive Wistar Kyoto rat s train.* Behavioural Brain Research, 2013. 253: p. 310-317.
36. A L Ackerman, F C Jellison, U J Lee, et al., *The Glt1 glutamate receptor mediates the establishment and perpetuatio n of chronic visceral pain in an animal model of stress-induced bladde r hyperalgesia.* American Journal of Physiology. Renal Physiology, 2016. 310(7): p. F628-F636.
37. B M. Santos, G C Nascimento, C P Capel, et al., *Sex differences and the role of ovarian hormones in site-specific noci ception of SHR.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2019. 317(2): p. R223-R231.
38. D Bleakman, A Alt and E S Nisenbaum, *Glutamate receptors and pain.* Seminars in Cell & Developmental Biology, 2006. 17(5): p. 592-604.
39. J-H Li, J-L Yang, S-Q Wei, et al., *Contribution of central sensitization to stress-induced spreading hype ralgesia in rats with orofacial inflammation.* Molecular Brain, 2020. 13(1): p. 106.
40. T Kameda, S Fukui, R Tominaga, et al., *Brain Metabolite Changes in the Anterior Cingulate Cortex of Chronic L ow Back Pain Patients and Correlations Between Metabolites and Psychol ogical State.* The Clinical Journal of Pain, 2018. 34(7): p. 657-663.
41. K Lv, W Song, R Tang, et al., *Neurotransmitter alterations in the anterior cingulate cortex in Crohn 's disease patients with abdominal pain: A preliminary MR spectroscopy study.* NeuroImage. Clinical, 2018. 20: p. 793-799.
42. S Jarrin, A Pandit, M Roche, et al., *Differential Role of Anterior Cingulate Cortical Glutamatergic Neurons in Pain-Related Aversion Learning and Nociceptive Behaviors in Male a nd Female Rats.* Frontiers in Behavioral Neuroscience, 2020. 14: p. 139.
43. A R Malik and T E Willnow, *Excitatory Amino Acid Transporters in Physiology and Disorders of the Central Nervous System.* International Journal of Molecular Sciences, 2019. 20(22): p. E5671.
44. R D Moloney, J Sajjad, T Foley, et al., *Estrous cycle influences excitatory amino acid transport and visceral pain sensitivity in the rat: effects of early-life stress.* Biology of Sex Differences, 2016. 7: p. 33.
45. E J Videlock, W Shih, M Adeyemo, et al., *The effect of sex and irritable bowel syndrome on HPA axis response an d peripheral glucocorticoid receptor expression.* Psychoneuroendocrinology, 2016. 69: p. 67-76.
46. D Li, R Liu, M Wang, et al., *3β-Hydroxysteroid dehydrogenase expressed by gut microbes degrades tes tosterone and is linked to depression in males.* Cell Host & Microbe, 2022. 30(3): p. 329-339.e5.
47. D Li, T Sun, Y Tong, et al., *Gut-microbiome-expressed 3β-hydroxysteroid dehydrogenase degrades estr adiol and is linked to depression in premenopausal females.* Cell Metabolism, 2023. 35(4): p. 685-694.e5.
48. N Goel, T J Philippe, J Chang, et al., *Cellular and serotonergic correlates of habituated neuroendocrine resp onses in male and female rats.* Psychoneuroendocrinology, 2022. 136: p. 105599.
49. E M Power and K J Iremonger, *Plasticity of intrinsic excitability across the estrous cycle in hypot halamic CRH neurons.* Scientific Reports, 2021. 11(1): p. 16700.

50. M M Heitkemper and L Chang, *Do fluctuations in ovarian hormones affect gastrointestinal symptoms i n women with irritable bowel syndrome?* Gender Medicine, 2009. 6 Suppl 2: p. 152-167.
51. P G Mermelstein, *Membrane-localised oestrogen receptor alpha and beta influence neurona l activity through activation of metabotropic glutamate receptors.* Journal of Neuroendocrinology, 2009. 21(4): p. 257-262.
52. X Xiao, Y Yang, Y Zhang, et al., *Estrogen in the anterior cingulate cortex contributes to pain-related aversion.* Cerebral Cortex (New York, N.Y.: 1991), 2013. 23(9): p. 2190-2203.
53. B Tang, Y Ji and R J Traub, *Estrogen alters spinal NMDA receptor activity via a PKA signaling path way in a visceral pain model in the rat.* Pain, 2008. 137(3): p. 540-549.
54. Y Ji, G Bai, D Y Cao, et al., *Estradiol modulates visceral hyperalgesia by increasing thoracolumbar spinal GluN2B subunit activity in female rats.* Neurogastroenterology and Motility: The Official Journal of the Europe an Gastrointestinal Motility Society, 2015. 27(6): p. 775-786.
55. L-H Sun, W-X Zhang, Q Xu, et al., *Estrogen modulation of visceral pain.* Journal of Zhejiang University. Science. B, 2019. 20(8): p. 628-636.
56. L López-Gómez, Y López-Tofiño and R Abalo, *Dependency on sex and stimulus quality of nociceptive behavior in a co nscious visceral pain rat model.* Neuroscience Letters, 2021. 746: p. 135667.
57. S L Byers, M V Wiles, S L Dunn, et al., *Mouse estrous cycle identification tool and images.* PloS One, 2012. 7(4): p. e35538.
58. Y Ji, B Tang and R J Traub, *The visceromotor response to colorectal distention fluctuates with the estrous cycle in rats.* Neuroscience, 2008. 154(4): p. 1562-1567.
59. A P Thomazi, G F R S Godinho, J M Rodrigues, et al., *Ontogenetic profile of glutamate uptake in brain structures slices fro m rats: sensitivity to guanosine.* Mechanisms of Ageing and Development, 2004. 125(7): p. 475-481.
60. J Sajjad, V D Felice, A V Golubeva, et al., *Sex-dependent activity of the spinal excitatory amino acid transporter : Role of estrous cycle.* Neuroscience, 2016. 333: p. 311-319.
61. S Kameoka, D Motooka, S Watanabe, et al., *Benchmark of 16S rRNA gene amplicon sequencing using Japanese gut microbiome data from the V1–V2 and V3–V4 primer sets.* BMC Genomics, 2021. 22(1): p. 527.
62. J G Caporaso, J Kuczynski, J Stombaugh, et al., *QIIME allows analysis of high-throughput community sequencing data.* Nature Methods, 2010. 7(5): p. 335-336.
63. Y Benjamini and Y Hochberg, *Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing.* Journal of the Royal Statistical Society: Series B (Methodological), 1995. 57(1): p. 289-300.
64. J Oksanen, F G Blanchet, R Kindt, et al., *vegan: Community Ecology Package*. 2012.
65. Y Ji, B Tang, D-Y Cao, et al., *Sex differences in spinal processing of transient and inflammatory col orectal stimuli in the rat.* Pain, 2012. 153(9): p. 1965-1973.
66. B A Vogt, *Pain and emotion interactions in subregions of the cingulate gyrus.* Nature Reviews. Neuroscience, 2005. 6(7): p. 533-544.
67. A J Shackman, T V Salomons, H A Slagter, et al., *The integration of negative affect, pain and cognitive control in the cingulate cortex.* Nature Reviews. Neuroscience, 2011. 12(3): p. 154-167.
68. X Xiao, M Ding and Y-Q Zhang, *Role of the Anterior Cingulate Cortex in Translational Pain Research.* Neuroscience Bulletin, 2021. 37(3): p. 405-422.

69. J P Johansen, H L Fields and B H Manning, *The affective component of pain in rodents: direct evidence for a cont ribution of the anterior cingulate cortex.* Proceedings of the National Academy of Sciences of the United States o f America, 2001. 98(14): p. 8077-8082.
70. J Fan, X Wu, Z Cao, et al., *Up-regulation of anterior cingulate cortex NR2B receptors contributes to visceral pain responses in rats.* Gastroenterology, 2009. 136(5): p. 1732-1740.e3.
71. B R Godlewska, M Browning, R Norbury, et al., *Predicting Treatment Response in Depression: The Role of Anterior Cing ulate Cortex.* The International Journal of Neuropsychopharmacology, 2018. 21(11): p. 988-996.
72. S C Caetano, S Kaur, P Brambilla, et al., *Smaller cingulate volumes in unipolar depressed patients.* Biological Psychiatry, 2006. 59(8): p. 702-706.
73. D C Shields, W Asaad, E N Eskandar, et al., *Prospective assessment of stereotactic ablative surgery for intractabl e major depression.* Biological Psychiatry, 2008. 64(6): p. 449-454.
74. W C Drevets, D Ongür and J L Price, *Neuroimaging abnormalities in the subgenual prefrontal cortex: implica tions for the pathophysiology of familial mood disorders.* Molecular Psychiatry, 1998. 3(3): p. 220-226, 190-191.
75. G Rajkowska and J J Miguel-Hidalgo, *Gliogenesis and Glial Pathology in Depression.* CNS & neurological disorders drug targets, 2007. 6(3): p. 219-233.
76. M J Taylor, S Selvaraj, R Norbury, et al., *Normal glutamate but elevated myo-inositol in anterior cingulate corte x in recovered depressed patients.* Journal of Affective Disorders, 2009. 119(1-3): p. 186-189.
77. L Colic, F Düring, D Denzel, et al., *Rostral Anterior Cingulate Glutamine/Glutamate Disbalance in Major Dep ressive Disorder Depends on Symptom Severity.* Biological Psychiatry: Cognitive Neuroscience and Neuroimaging, 2019. 4(12): p. 1049-1058.
78. S J Millard, J S Lum, F Fernandez, et al., *The effects of perinatal fluoxetine exposure on emotionality behaviour s and cortical and hippocampal glutamatergic receptors in female Sprag ue-Dawley and Wistar-Kyoto rats.* Progress in Neuro-Psychopharmacology & Biological Psychiatry, 2021. 108: p. 110174.
79. P Luczynski, M Tramullas, M Viola, et al., *Microbiota regulates visceral pain in the mouse.* eLife, 2017. 6: p. e25887.
80. Y Zhang, Q Fan, Y Hou, et al., *Bacteroides species differentially modulate depression-like behavior v ia gut-brain metabolic signaling.* Brain, Behavior, and Immunity, 2022. 102: p. 11-22.
81. P Strandwitz, K H Kim, D Terekhova, et al., *GABA Modulating Bacteria of the Human Gut Microbiota.* Nature microbiology, 2019. 4(3): p. 396-403.
82. C A Simpson, A Mu, N Haslam, et al., *Feeling down? A systematic review of the gut microbiota in anxiety/dep ression and irritable bowel syndrome.* Journal of Affective Disorders, 2020. 266: p. 429-446.
83. H Li, Y Xiang, Z Zhu, et al., *Rifaximin-mediated gut microbiota regulation modulates the function of microglia and protects against CUMS-induced depression-like behaviors in adolescent rat.* Journal of Neuroinflammation, 2021. 18: p. 254.
84. M I Pinto-Sanchez, G B Hall, K Ghajar, et al., *Probiotic Bifidobacterium longum NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome.* Gastroenterology, 2017. 153(2): p. 448-459.e8.

85. Y He, Y Tan, J Zhu, et al., *Effect of sodium butyrate regulating IRAK1 (interleukin-1 receptor-associated kinase 1) on visceral hypersensitivity in irritable bowel syndrome and its mechanism.* Bioengineered, 2021. 12(1): p. 1436-1444.
86. Y Tang, J Du, H Wu, et al., *Potential Therapeutic Effects of Short-Chain Fatty Acids on Chronic Pain.* Curr Neuropharmacol, 2024. 22(2): p. 191-203.
87. K Sato, N Matsuki, Y Ohno, et al., *Estrogens inhibit l-glutamate uptake activity of astrocytes via membra ne estrogen receptor alpha.* Journal of Neurochemistry, 2003. 86(6): p. 1498-1505.
88. X D'Anglemont de Tassigny, C Campagne, S Steculorum, et al., *Estradiol induces physical association of neuronal nitric oxide synthase with NMDA receptor and promotes nitric oxide formation via estrogen receptor activation in primary neuronal cultures.* Journal of Neurochemistry, 2009. 109(1): p. 214-224.
89. M S Jansen, S C Nagel, P J Miranda, et al., *Short-chain fatty acids enhance nuclear receptor activity through mito gen-activated protein kinase activation and histone deacetylase inhibi tion.* Proceedings of the National Academy of Sciences of the United States o f America, 2004. 101(18): p. 7199-7204.