Characterising Gut Dysbiosis in Rodent Models Relevant to Psychiatric and Neurodevelopmental Disorders

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MSc (research) Biomedical Science Thesis

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I, Jodie Ellis, confirm that the work presented in this thesis is my own and has not been submitted in substantially the same form for the award of a higher degree elsewhere. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

Signed: J. Ellis

Submitted in part fulfilment of the requirements for the degree of Research Masters

Jodie Ellis, Biomedical Science BSc (Hons), Characterising Gut Dysbiosis in Rodent Models Relevant to Psychiatric and Neurodevelopmental Disorders, Research Masters Thesis, December 2019

Abstract

Title: Characterising Gut Dysbiosis in Rodent Models Relevant to Psychiatric and Neurodevelopmental Disorders

Background: The microbiota is central to gut homeostasis and overall health, with dysbiosis linked to multiple morbidities. The gut and brain are linked via multiple, bidirectional, communication mechanisms including the vagus nerve, immune system, and neurotransmitter metabolism. Increased attention has been given to the microbiota's impact on the brain and its potential role in psychiatric and neurodevelopmental disorders (PNDs), such as schizophrenia and autism. We aim to investigate the role of the microbiome and gut dysfunction in these disorders to further understand the potential mechanisms involved. The Phencyclidine (PCP) and *Neurexin1α* heterozygous (*Nrxn1α*$^{+/-}$) mouse models are relevant to schizophrenia and autism.

Materials and Methods: Bacterial DNA, extracted from the intestinal contents of PCP and *Nrxn1α*$^{+/-}$ mice, were subject to community profiling by Denaturing Gradient Gel Electrophoresis (DGGE). Gut tissue were analysed histologically to determine the impact on gut architecture and EECs.

Results: Subchronic PCP treatment significantly increased the depth of the muscle layer in the colon but had no effect on gut architecture or the microbiome composition. *Nrxn1α*$^{+/-}$ significantly decreased the depth of the muscle layer in the colon and caecum in mice of both sexes. *Nrxn1α*$^{+/-}$ impacted on gut architecture in female but not in male mice. By contrast, *Nrxn1α*$^{+/-}$ selectively decreased GLP-1 positive EEC number in males. *Nrxn1α*$^{+/-}$ also significantly affected gut microbiome composition. Liraglutide did not reverse the impact of *Nrxn1α*$^{+/-}$ on the gut. However, Liraglutide treatment selectively decreased EEC number in the caecum of *Nrxn1α*$^{+/-}$ males. Liraglutide treatment also significantly decreased goblet cell size in the colon, and increased goblet cell size in the caecum of male mice, independent of

genotype. Liraglutide-treated mice had a significantly different microbiome composition in the mucus layer of the colon and caecum.

Conclusions: As PCP had no effect on the gut architecture or microbiome, it is unlikely that it models the gut dysfunction seen in schizophrenia. Therefore, the behaviour changes in PCP mice are independent of effects on the gut. *Nrxn1α*$^{+/-}$ impacted on the gut and microbiome which may be relevant to the behavioural changes seen in *Nrxn1α*$^{+/-}$ mice. Changes to the gut and microbiome may be one mechanism by which *Nrxn1α*$^{+/-}$ increases the risk of developing PNDs. Liraglutide did not reverse the impact of *Nrxn1α*$^{+/-}$ on the gut. However, Liraglutide did modify gut architecture and the microbiome which could be relevant to the use of the drug in type II diabetes.

Abbreviations

16S ribosomal RNA (rRNA)

4’,6-diamidino-2-phenylindole (DAPI)

Acetylcholine (Ach)

Alcian blue (AB)

Ammonium persulphate (APS)

Antibody raised against CgA (anti-CgA)

Antigen raised against Glucagon-like peptide-1 (anti-GLP-1)

Attention-deficit hypersensitivity disorder (ADHD)

Autism spectrum disorders (ASD)

Bacterial operational taxonomic units (OTU’s)

Blood-brain-barrier (BBB)

Body mass index (BMI)

Bovine Serum Albumin (BSA)

Brain-derived neurotropic factor (BDNF)

Catechol-O-Methyl Transferase (*COMT*)

Central nervous system (CNS)

Cerebrospinal fluid (CSF)

Chromogranin A (CgA)

Corticotropin-releasing factor (CRF)

C-reactive protein (CRP)

Denaturing Gradient Gel Electrophoresis (DGGE)

Dendritic cells (DC)

Dibutylphthalate Polystyrene Xylene (DPX)

Dopamine (DA)

Enteroendocrine cells (EECs)

Epidermal growth factor (EGF)

Epinephrine (E)

Excitatory postsynaptic current (EPSC)

Fc Fragment of IgG Binding Protein (FCGBP)

Gastrointestinal tract (GI)

Genome-wide analysis studies (GWAS)

Germ-free (GF)

Glucagon gene (GCG)

Glucagon-like peptide-1 (GLP-1)

Glucagon-like peptide-2 (GLP-2)

G-protein coupled receptors (GPCR's)

Hydrochloric acid (HCL)

Hypothalamic–pituitary–adrenal (HPA)

Interferon (IFN)

Interleukin (IL)

Interleukin 1-beta (IL1β)

Irritable bowel syndrome (IBS)

Mitogen-activated protein kinase (MAPK)

Mucin 2, Oligomeric Mucus/Gel-Forming (MUC2)

Natural killer cells (NK cells)

Neurexin gene *(Nrxn)*

Norepinephrine (NE)

Nrxn1α heterozygous deletions (*Nrxn1α*$^{+/-}$)

Peptide YY (PYY)

Peroxidase-Antiperoxidase (PAP)

Phencyclidine (PCP)

Phosphate-buffered saline (PBS)

Protein kinase B (Akt)

Psychiatric and neurodevelopment disorders (PNDs)

Regulatory T-cells (Tregs)

Serotonin (5-HT)

Short chain fatty acids (SCFAs)

Small nucleotide polymorphisms (SNP's)

Tetramethylethylenediamine (TEMED)

Tris-acetate-EDTA (TAE)

Tumour necrosis factor-alpha (TNFα)

Contents

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1. Literature Review

1.1 Architecture of the gut

The gastrointestinal (GI) tract is an organ present within humans and animals which consists of the mouth, oesophagus, stomach, small intestine (duodenum, jejunum and ileum), large intestine (caecum, colon and rectum), and anus (Figure 1, Newman, 2018). 90% of food digestion and absorption occurs in the small intestine; the internal walls of the small intestine are covered in villi and microvilli which increase the surface area available to absorb nutrients. The other 10% occurs in the stomach and large intestine. The main function of the large intestine is to absorb water from indigestible food and eliminate waste material from the body; this takes place in the colon. In humans, the caecum has no function although in mice it is an important site for fermenting plant materials as well as producing vitamin K and B, which mice reabsorb through coprophagy (Ngugen et al, 2015).

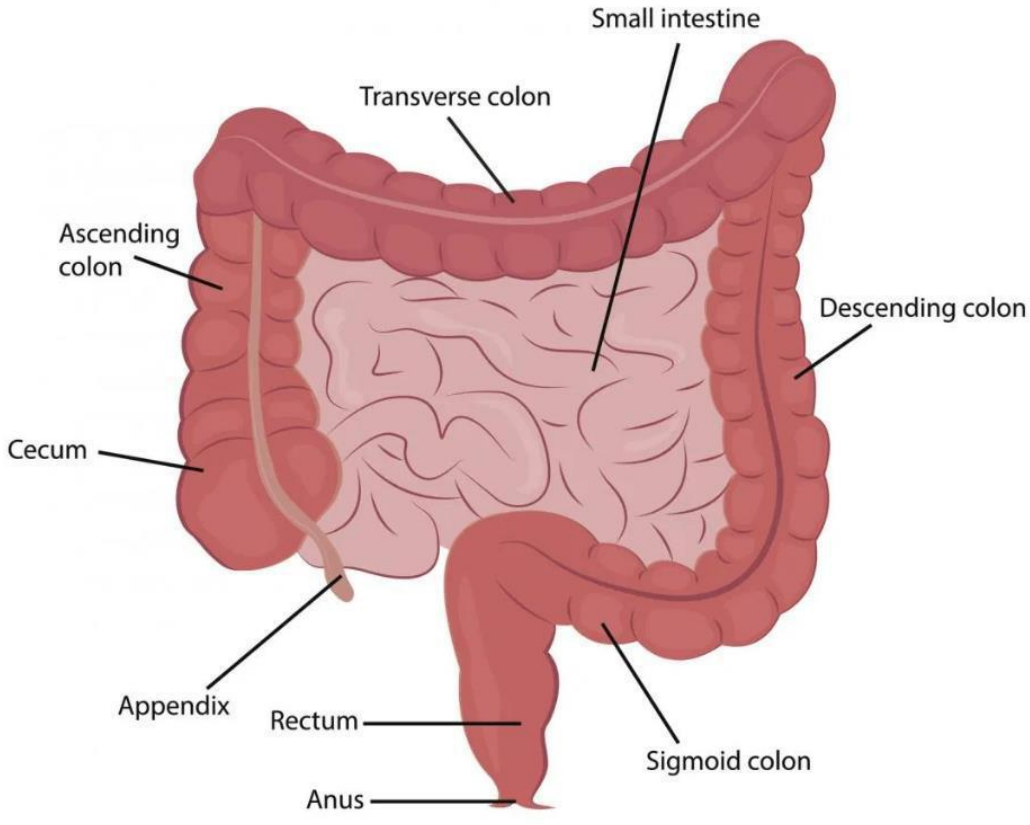


Figure 1: Labelled diagram of the human intestinal tract. Diagram shows location of the colon and caecum (appendix) which we used in our study for analysis. (Newman, 2018).

1.2 Types of cells produced in the intestine

The surface of the intestinal epithelium is renewed from stem cell populations at the base of the crypt and has a cell turnover between 3-7 days. These stem cells produce several cell types within the gut, including goblet cells, enteroendocrine cells (EECs), enterocytes, Paneth cells and tuft cells (Birchenough et al, 2015) which have important roles within the GI tract.

1.2.1 Goblet cells

Goblet cells are specialised cells that synthesise and secrete mucus, mainly Mucin 2, Oligomeric Mucus/Gel-Forming (MUC2) (Pelaseyed et al, 2014). Mucus has vital roles in the GI tract which includes protecting cell linings against the surrounding acidic environment and reducing intestinal inflammation by decreasing the interactions of bacteria with epithelial cells (Hansson, 2012). Intestinal mucus is essential for the harbouring of large quantities of bacteria in the gut and is mainly composed of the mucin, MUC2 (Johansson et al, 2011). An absence of MUC2 can trigger inflammation which impacts the permeability of the separation barrier, thus encouraging the translocation of gut bacteria into the systemic circulation (Severence et al, 2016). The antibacterial peptides and proteins secreted from Paneth cells and enterocytes into the mucus also play a role in keeping gut bacteria separated from the epithelial cells (Elphick and Mahida, 2005).

1.2.2 EECs

EECs which are produced from the same pluripotent stem cell population as goblet cells, have various roles depending on their location within the gut although they only comprise 1% of the total epithelial cell population (Stirnini et al, 2010). They produce hormones and peptides in response to various stimuli which are either released into the bloodstream, used as local messengers, or transmitted to the enteric nervous system (Latorre et al, 2016). The numerous types include enterochromaffin cells, cholecystokinin-secreting cells, secretin-secreting S cells, gastric inhibitory polypeptide-secreting cells, motilin-secreting M cells and neurotensin-secreting N cells (Rindi et al, 2004). EECs are restricted to the mucosa with greater diversity in the small intestine. Chromogranin A (CgA), a matrix-soluble glycoprotein, is a component of secretory vesicles which EECs possess and is commonly

used as a marker to reflect their presence and function in the gut. Immunohistochemical studies show that EECs stain positively for CgA in all regions of the GI tract (Schmid et al, 1989).

Enterochromaffin cells are the most abundant EEC and comprise over 70% of the total EEC population in the proximal large bowel and 40% in the rectum (Gunawardene et al, 2011). Their main secretory product is serotonin (5-HT), synthesised by the hydroxylation and decarboxylation of tryptophan (Ahlman & Nilsson 2003). 5-HT is important for intestinal motility, intestinal secretion, visceral sensation and appetite. Other secretory products by EECs include glucagon-like peptide-1 (GLP-1), omatostatin (SST), peptide YY (PYY), glucagon-like peptide-2 (GLP-2), glicentin and oxyntomodulin (OXM).

GLP-1 is a major contributor to the incretin effect, where gut hormones stimulate release of insulin from the pancreas in response to the ingestion of glucose. GLP-1 binds to its beta cell receptors on the islets of Langerhans which stimulates insulin transcription, biosynthesis and release. In addition, GLP-1 stimulates beta cell differentiation and proliferation (Gunawardene et al, 2011). GLP-1 will be discussed in more detail later.

1.3 The Gut Microbiome

The microbiome is the combined diversity of the microorganisms present in a particular environment. The gut microbiota is a diverse, complex community of microorganisms which are present throughout the digestive system of humans and animals, consisting of approximately 10^{14} cells (Sender et al, 2016). The large intestine houses the largest community of bacteria in the body, which is composed mainly of obligate anaerobes (Canny and McCormick, 2008). The most common bacteria present belong to the genus Bacteroides which include anaerobic gram-positive cocci, such as *Peptostreptococcus* sp., *Eubacterium* sp., *Lactobacillus* sp., and *Clostridium* sp. (Canny and McCormick, 2008). Recent examinations of large bowel biopsies have confirmed Bacteroides as the dominant genus (Canny and McCormick, 2008).

The microbiome plays an important role in disease, immunity and brain function. It protects the host by protecting against pathogens such as *Streptococcus pneumoniae* (Schuijt et al, 2015), has a primary role in metabolising dietary nutrients and drugs (Singh et al, 2017), and influences the absorption and distribution of dietary fat (Martinez-Guryn et al, 2018). A potential role for the microbiota in psychiatric and neurodevelopmental disorders (PND's) has recently become an emerging topic of interest (Warner BB, 2019), where the microbial composition of the gut could be a target area of treatment. PND's include depression, anxiety, schizophrenia and autism. The main PND's of interest in this project are schizophrenia and autism.

1.3.1 The Gut Microbiome and the Gut-Brain Axis

The microbiota plays a major role in the bidirectional communication between the GI tract and the central nervous system (CNS). There are complex levels of communication between the intestinal microbiota and the brain and these interactions modulate the functioning of the immune system (El Aidy et al, 2015) and the CNS (McMurran et al, 2019). The gut-brain axis is composed of the CNS, sympathetic and parasympathetic nervous systems and the enteric nervous system, neuroendocrine and neuroimmune systems (El Aidy et al, 2015). The bidirectional communication between the microbiome and the CNS influences stress reactivity (Wiley et al, 2017), inflammation (Ma et al, 2019) and the pathogenesis of a number of disorders including mood disorder, autism spectrum disorders (ASD), attention-deficit hypersensitivity disorder (ADHD) and obesity (Petra et al, 2015).

Deviations in the synergistic relationship between the host and their microbiome can potentially exaggerate these complications.

The gut interacts with the brain through several mechanisms. These include the production, expression and turnover of neurotransmitters such as 5-HT and gamma-aminobutyric acid (GABA) (Martin et al, 2018), tryptophan metabolism (Liang et al, 2018), the protection of the intestinal barrier and tight junction integrity (Carabotti et al, 2015), producing bacterial metabolites (Kim et al, 2018) and mucosal immune regulation (Powell et al, 2017). Furthermore, the brain also interacts with the gut microbiota. The main mechanisms include alterations in the mucus and biofilm production, motility (Mayer et al, 2015), intestinal permeability and immune function (Carabotti et al, 2015). The GI tract and the brain are very closely associated, and communication mainly occurs through the vagus nerve. The gut microbiota can affect the immune system directly via activation of the vagus nerve, in turn triggering bidirectional communication with the CNS (Rogers et al, 2016). (Figure 2, Martin et al, 2018).

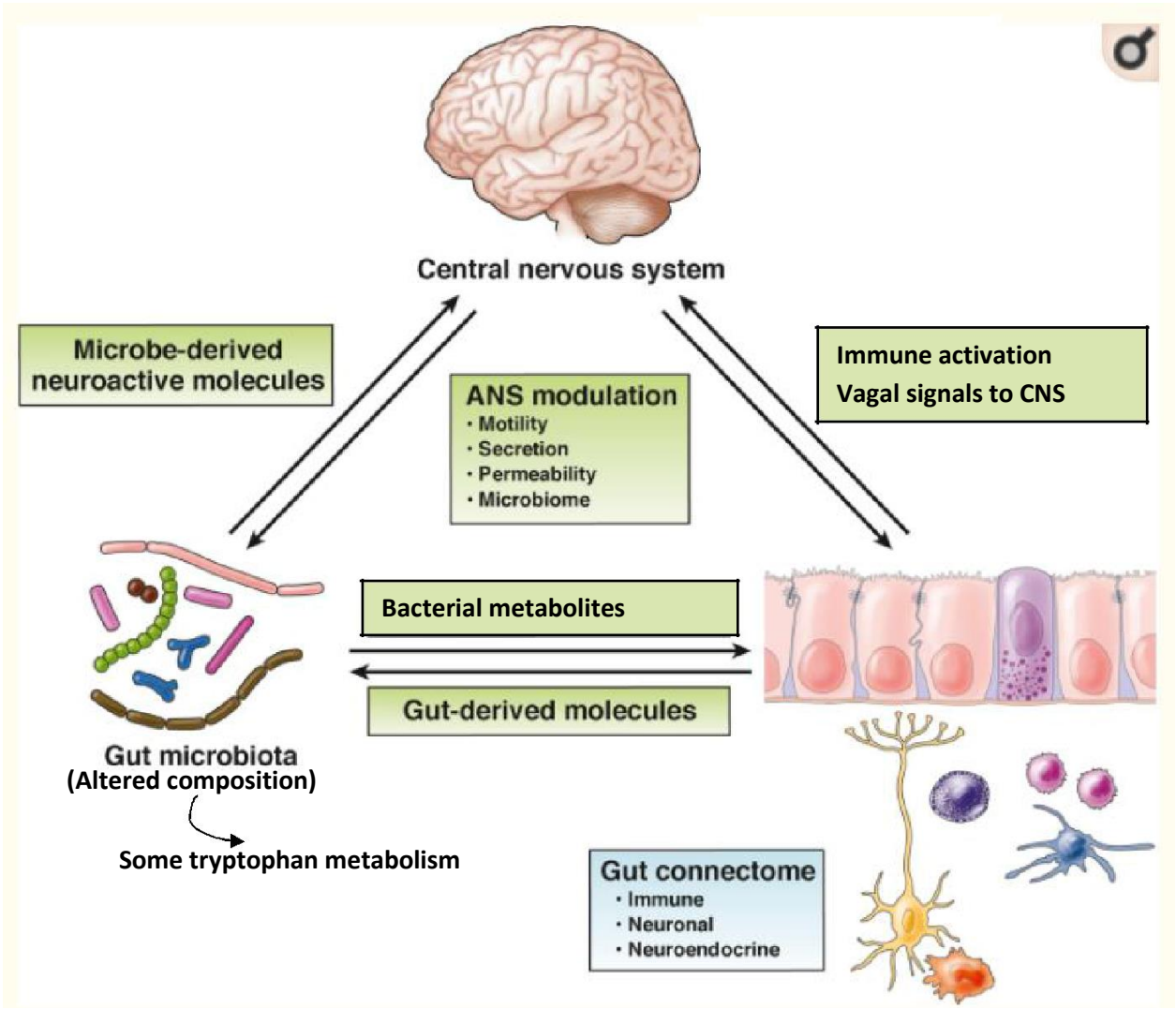


Figure 2: Interactions between the gut microbiota and the CNS. The gut interacts with the CNS through bacterial metabolites, immune activation, tryptophan metabolism and neural pathways (vagus nerve). The CNS interacts with the microbiota through modulating motility, secretion, intestinal permeability and microbiome composition. Diagram was adapted from Martin et al, 2018.

Bacteria also ferment components from the diet which leads to the production of short chain fatty acids (SCFA's) inside the colon. These include acetate, butyrate, and propionate and can interact with immune and metabolic pathways (Joseph et al, 2017). SCFA's can have immunomodulatory properties and can interact with nerve cells by stimulating the sympathetic autonomic nervous system which occurs via G-protein coupled receptors (GPCR's) 41 and 43 (Kimura et al, 2011). One study investigated the effects of SCFA's and their receptors on the immune response; GPCR 41 and 43 knockout mice exhibited a slower immune response against *Citrobacter rodentium* infection (Kim et al, 2013). In addition,

healthy mice fed with SCFA's had activated GPCR41 and GPCR43 which lead to activated extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase (MAPK) signalling pathways in epithelial cells, this induced the production of chemokines and cytokines during immune responses (Kim et al, 2013). This suggests SCFA's and their receptor pathways mediate protective immunity in mice. SCFA's also cross the BBB which affects brain behaviour (Macfabe et al, 20012). For example, butyrate specifically has been shown to act as a histone deacetylase inhibitor, increasing the expression of genes related to neural regeneration and plasticity (Koh et al, 2016). SCFA's can also promote the growth of enterocytes (Alexander et al, 2019). Finally, SCFA's can regulate the release of gut peptides from EECs such as GLP-1 (Covasa et al, 2019). For example, ingestion of fructooligosaccharides which produces high levels of luminal SCFA'S induces the proliferation of L-cells which expresses GLP-1 (Covasa et al, 2019), and GLP-1 can induce metabolic actions by interacting with its receptors expressed on nerve cells in the gut and the brain (Cabou and Burcelin, 2011).

1.3.2 Establishment of the Gut Microbiome and Associations with PND's During Development

Throughout life the microbiome changes and establishes a mutual beneficial relationship with the host. Establishment of the microbiota occurs in parallel with neurodevelopment and both have critical development windows which are sensitive to disruption (Tognini, 2017). Events during the initial colonisation of the microbiome during early life can have impact on the development of the immune system, increasing the susceptibility of developing disease in later life (Gensollen et al, 2016). A study in mice showed that immune development was significantly different in mice conventionalised in adulthood, suggesting colonisation during development is required to establish mucosal immune homeostasis in a way which cannot be achieved in adulthood (El Aidy et al, 2013).

Preclinical studies have shown that in early life the absence of the microbiota significantly affects hippocampal monoamine gene-regulation which has been associated with anxiolytic behaviours (Pan et al, 2019). Preclinical studies using germ-free (GF) mice suggested the ability of early-life microbiota to influence neurodevelopment has long-lasting effects on neural function; these GF mice had changes to the brain including alterations in

monoaminergic transmission and behavioural changes such as anxiety (Foster and McVey Neufeld, 2013). In addition, the use of probiotics on GF mice significantly increases levels of 5-HT and dopamine (DA) in the striatum which induces changes to anxiety-like and depressive-like behaviours, suggesting the expression of monoamine neurotransmitter-related genes is impacted by the absence of commensal microbiota during early life (Martin et al, 2018).

There have also been links between events in prenatal life and the development of neurodevelopment disorders such as autism, ADHD and schizophrenia (Neufeld et al, 2011). Specifically, maternal prepregnant body mass index (BMI) (Schaefer et al, 2010), maternal infection (Blomstrom et al, 2016), and prenatal stress particularly in male offspring (Fineberg et al, 2016) have all been shown to impact on the likelihood of developing autism and schizophrenia. Studies in mice support this by suggesting the exposure to microbial pathogens such as *C. rodentium* during similar developmental periods results in behavioural abnormalities including anxiety-like behaviour and impaired cognitive function (Lyte et al, 2006). It was also shown that the complete absence of a conventional microbiota leads to decreased anxiety-like behaviour in these rodents (Neufeld et al, 2011). However, the measurement of anxiety can be variable based on what we consider to be anxious behaviour in mice.

1.4 The GI Tract and the Brain

Microorganisms within the gut have the ability to influence behaviour, possibly because they can synthesise and release neurochemicals which have an identical structure to those produced by the hosts nervous system (Lyte, 2013). The key neurotransmitters produced by enteric bacteria are norepinephrine (NE), epinephrine (E), DA, and 5-HT which are important for healthy brain function. For example, NE is important for alertness and the formation and retrieval of memory, E stimulates heart rate and increases blood pressure in strong emotional situations, DA is important for motor control, motivation and reward, and 5-HT regulates mood, appetite and sleep. In addition to the synthesis of neurochemicals, enteric bacteria possess neurotransmitter receptors in which they respond to (Mittal et al, 2017). Studies have shown that these binding sites can influence the function of components of the microbiota and increase the predisposition to inflammatory and infection stimuli (Hughes and Sperandio, 2008). For example, NE promotes the growth of *Eschereschia coli* (Burton et al, 2002); 5-HT promotes growth of *Turicibacter* and *Clostridia* (Fung et al, 2019). DA can downregulate virulence gene transcription from *Salmonelle enterica* (Yang et al, 2019), and an overall downregulation of these neurotransmitters can cause a variety of gastrointestinal morbidities such as irritable bowel syndrome (IBS) (Mittal et al, 2017).

Gut bacteria can produce immune factors causing release of cytokines and inflammatory mediators from the host which have targets within the CNS and the enteric nervous system. Enteric bacteria interact both locally with intestinal cells and directly with the CNS through neuroendocrine and metabolic pathways. Molecules produced which act as local neurotransmitters include gamma-aminobutyric acid (GABA), 5-HT, melatonin, histamine and acetylcholine (Ach) (Iyer et al, 2004). Enteric bacteria also produce metabolites including butyric acid, propionic acid and acetic acid, all which can stimulate the sympathetic nervous system, mucosal 5-HT release and influence the process of learning and memory (Carabotti et al, 2015).

Studies on GF animals have shown that bacterial colonization of the gut is central to development and maturation of both enteric and CNS (Barbara et al, 2005), and absence of these bacteria is linked to an altered expression and turnover of neurotransmitters in both systems. Furthermore, there is a high rate of comorbidity between gastrointestinal dysfunction and psychiatric illnesses (Rogers et al, 2016).

1.5 The Aetiology of Schizophrenia

Schizophrenia is a long-term medical disorder which affects approximately 1% of the population and is slightly more common in males than females. It is characterised by delusions and hallucinations (positive symptoms), anhedonia, affective flattening and avolition (negative symptoms) (Nomura et al, 2016). There are a few different hypotheses on what causes the symptoms of schizophrenia as outlined below.

1.5.1 Dopamine Hypothesis

The DA hypothesis of schizophrenia is caused by DA dysregulation; there is an increase in DA synthesis and release into the dorsal striatum (Petty et al, 2019) and mesolimbic pathway (Nikolaus et al, 2018) which leads to the positive symptoms of the disorder. There are dopaminergic deficits in the prefrontal brain regions which may contribute to the negative symptoms (McCutcheon et al, 2019) and cognitive deficits (Brisch et al, 2014). An example study showed higher DA receptor D2 density in the caudate nucleus was associated with poorer performance in cognitive tasks which involved corticostriatal pathways (Hirvonen et al, 2005). This is further supported by neuroimaging which represents a higher density of D2 receptors in the brains of schizophrenic patients in comparison to healthy controls (McGuire et al, 2008). DA is a key target for drug development; all common treatments currently used in the treatment of schizophrenia involve antidopaminergic, specifically DA 2 receptor, mechanisms (Szlachta et al, 2018).

1.5.2 Glutamate Hypothesis

The glutamate hypothesis of schizophrenia suggests that dopaminergic dysfunction is caused by an underlying glutamatergic dysfunction in the disorder, where the glutaminergic hypofunction in corticostriatal projections causes changes in DA concentrations (Howes et al 2015). The glutamate receptor, N-methyl-D-aspartate receptor (NMDA-R), plays a vital role in the pathophysiology of schizophrenia as NMDA-R antagonists produce schizophrenia-like symptoms (Amat-Foraster et al, 2019), and the enhancement of NMDA-R function reduces schizophrenias negative symptoms and improves cognition (Coyle, 2012). Genetic studies have shown NMDA-R hypofunction to be correlated with the symptoms of schizophrenia (Goff and Coyle, 2001). In addition, there is the GABAergic hypothesis of schizophrenia

which is evidenced by altered expressions of the key enzymes for GABA biosynthesis, the 67 and 65kDA isoforms of glutamic acid decarboxylase (GAD67 and GAD65), as is shown by altered expression levels in brain post-mortems of those diagnosed with schizophrenia (de Jonge et al, 2017). The calcium-binding proteins, parvalbumin and calbindin, can be used as markers to locate cortical GABAergic interneurons. Parvalbumin-expressing neurons are likely a key location for the pathophysiology which underlies schizophrenia and have shown to be implicated such cases (Kaar et al, 2019). One study showed reduced or absent GAD67 mRNA in 50% of parvalbumin positive GABAergic cells (Woloszynowska-Fraser et al, 2017). There has also been reported increased calbindin mRNA in schizophrenia cases (Fung et al, 2014). Together, these show that dysfunctional glutamate receptors, and potentially GABAergic dysfunction, are key contributors to the pathogenesis of schizophrenia.

1.5.3 Genetic Predispositions

Certain genes increase the susceptibility to schizophrenia, although the most common genetic mutations only contribute a small amount to the increased predisposition of developing the disease. Here, not a single gene is responsible but rather a combination of the weak effects of several genes acting together. Example causative genes include *Catechol-O-Methyl Transferase (COMT)* because of its role in monoamine metabolism (Harrison and Weinberger, 2005); *Dysbindin*, whose expression is decreased in schizophrenia (Straub et al, 2002); and *Neuregulin 1* because of its involvement in neurodevelopment, regulation of glutamate and synaptic plasticity (Tosato et al, 2005). However, analysis of small nucleotide polymorphisms (SNP's) in the COMT gene suggested it is unlikely to play a significant role in schizophrenia development (Kong et al, 2011 and Bag, 2018). On the other hand, genetic association studies have revealed significant links between the *Neuregulin 1* gene and a higher susceptibility to developing schizophrenia (Yang et al, 2003), and a polymorphism in the *Dysbindin* gene (*DTNBP1*) has been correlated with schizophrenia amongst other anxiety related psychiatric disorders (Voisey et al, 2010).

Rarer genetic mutations including those on the *Neurexin (Nrxn)* gene pose a much greater risk of developing schizophrenia. The *Nrxns* (1-3) are a group of synaptic adhesion proteins which are involved in the formation and maintenance of synapses (Wright and Washbourne,

2011). They bind to neuroligins to form calcium ion dependant complexes at the synapses in the CNS which is required for effective neurotransmission (Missler et al, 2003). They also induce post-synaptic differentiation in glutaminergic synapses and regulate NMDA-R (Graf et al, 2004), where NMDA-R dysfunction is proposed to be a major contributor to schizophrenia (Coyle, 2012). The gene for *Nrxn1* is located on chromosome 2p16.3; it is one of the largest genes in the human genome (Rowen et al, 2002). Studies which have investigated copy number variation in schizophrenia have found several *Nrxn1α* heterozygous deletions (*Nrxn1α*$^{+/-}$) which are estimated to be present in 0.16% of affected individuals *(Kirov et al, 2009)*. Most *Nrxn1α*$^{+/-}$ deletions eliminate the promotor and exon regions of the gene, where exon deletions are highly correlated with schizophrenia (Kirov et al, 2009).

A microdeletion in chromosome 22 may be involved in a few schizophrenia cases and was identified in genome-wide analysis studies (GWAS); it increases the risk of developing the disorder by up to 30% in individuals with the deletion (Bassett et al, 2008). In a GWAS in 2014, 108 genetic loci were identified which were associated with schizophrenia; these genes are expressed in the brain and immune cells involved in adaptive immunity (Khandaker et al, 2015). This suggests the immune system plays a key role in the risk of developing schizophrenia.

1.5.4 Immune Hypothesis

The immune hypothesis of schizophrenia suggests that the onset of the disease is triggered by infectious agents from the environment which bring about an immune response, and this is supported by some clinical data. Peripheral immune cells in schizophrenic patients have been reported as abnormal. For example, a study showed schizophrenia cases to have increased numbers of natural killer (NK) cells, naïve B cells, C-X-C chemokine receptor type 5 memory T cells (CXCR5^{+}) and classical monocytes; and *decreased* numbers of dendritic cells (DC), Human Leukocyte Antigen - DR isotype (HLA-DR^{+}), regulatory T-cells (Tregs), and CD4^{+} memory T cells (Fernandez-Egea et al, 2016). Furthermore, epidemiological studies show an association of schizophrenia with autoimmunity and allergies (Eaton et al, 2006). An example risk factor is maternal infection during second-trimester pregnancy; a study

showed an elevated incidence of schizophrenia in the offspring following an influenza epidemic (Brown et al, 2019). Exposure to infections causes macrophages to produce inflammatory cytokines which signal through endothelial cells to change the structure of the tight junctions of the BBB thus causing increased permeability and alterations to brain structure and function (Pan et al, 2011). Inflammatory molecules such as interleukin 1-beta (IL1β) and tumour necrosis factor-alpha (TNFα) have repeatedly been shown to be elevated in schizophrenia cases as compared to healthy controls (Boerrigter et al, 2017). Adult schizophrenia has also been correlated with increased rate of infection. For example, *Toxoplasma gondii* which causes Toxoplasmosis infection, is associated with an increased risk of developing the disorder (Yolken et al, 2017). Another study found that *T. gondii* infection increased the risk in females only (Khademvatan et al, 2014). However, a study by De Campos-Carli et al, 2017, found no significant associations between the two.

1.5.5 Neurodevelopmental Hypothesis

The neurodevelopmental hypothesis postulates that schizophrenia is caused by effects during the development of the embryos brain which leads to defective neural connectivity which results in cognitive dysfunction in later life (Fatemi and Folsom, 2009). Brain image studies show ventricular enlargement, reductions in brain volume and changes of cortical thickness in schizophrenia patients, which are possible as a result of early impaired neurodevelopment (Ho et al, 2003).

1.5.6 Insulin Abnormalities

Aberrant insulin receptor function may contribute to the pathophysiology of schizophrenia. A study showed increased levels of insulin and insulin resistance in first-onset schizophrenic individuals (Arranz et al, 2004). Also, insulin receptor autophosphorylation and insulin-dependent protein kinase B (Akt) signalling were significantly downregulated in a group of 12 schizophrenic patients (Zhao et al, 2006). Akt mediates neuregulin-1 and dysbindin signalling (Li et al, 2003), two of the predisposition genes which may increase the risk of developing schizophrenia. Due to abnormal insulin signalling the life expectancy of schizophrenic patients is reduced due to the association with linked pathologies such as cardiovascular disorders and type II diabetes (Ryan and Thakore, 2002).

1.6 Schizophrenia and the Gut Microbiome

Emerging research suggests that the human microbiome plays a role in the development of schizophrenia and thus could potentially be a target of treatment. Due to the role of the microbiome in the development and functioning of the CNS, there is a strong indication that these microbes impact on cognitive function and patterns of behaviour. The gut microbiota produces key neurotransmitters which include 5-HT, produced by *Candida*, *Streptococcus*, *Escherichia* and *Enterococcus* species (Evrensel and Ceylan, 2015); DA, produced by *Bacillus* and *Serratia* (Galland, 2014); NE, produced by *Escherichia*, *Bacillus* and *Saccharomyces* species (Dinan et al, 2015); and Ach which is produced by *Lactobacillus* (Galland, 2014). Lactobacillus and Bifidobacterium also produce GABA (Pokusaeva et al, 2017) which is the main inhibitory neurotransmitter in the brain besides glutamate (Valenzuela et al, 2011) (see Table 1). A dysfunctional microbiome could therefore disrupt and permanently alter the levels of essential neurotransmitters required for normal cognitive function (Dinan et al, 2014).

Neurotransmitter	**Example bacteria which produce neurotransmitter**	**Reference**
5-HT	*Candida, Streptococcus, Escherichia,* and *Enterococcus*	Evrensel and Ceylan, 2015
DA	*Bacillus* and *Serratia*	Galland, 2014
NE	*Escherichia*, *Bacillus* and Saccharomyces	Dinan et al, 2015
Ach	*Lactobacillus*	Galland, 2014
GABA	*Lactobacillus, Bifidobacterium*	Pokusaeva et al, 2017

Table 1: Example bacteria which produce key the neurotransmitters in the gut. Table shows the types of bacteria which produce 5-HT, DA, NE, ACh and GABA.

The microbiome in schizophrenia is significantly altered. For example, it has been reported that schizophrenic patients have a significantly lower number of *Bifidobacterium, Escherichia coli* and *Lactobacillus*, and a higher abundance of *Clostridium coccoides* when compared to healthy controls (Cuomo et al, 2018). When the microbiota of schizophrenia patients was transplanted to GF mice, behaviours such as decreased swimming immobility and an exaggerated startle in response to high decibel tones was observed (Zheng et al, 2019). As levels of neurotransmitters such as 5-HT and DA are elevated in schizophrenia, it is possible that the altered microbiome affects the neurochemistry relevant to the pathophysiology of the disease.

1.6.1 Evidence for Gut Dysfunction in Schizophrenia

The bacteria in the microbiota may produce harmful toxins which compromise the integrity of the epithelial barrier of the intestine, thus allowing entry of harmful products into circulation (Hornig, 2013). These bacterial products may have neuroactive properties. It has been evidenced that there is structural damage to the GI barrier in schizophrenic patients (Severence et al, 2012). Markers of intestinal inflammation and bacterial translocation were examined in schizophrenic patients, including antibodies to *Saccharomyces cerevisiae* and soluble cluster of differentiation 14 (CD14). Both were elevated in recent onset, chronically affected and antipsychotic-naïve patients with schizophrenia (Severance et al, 2012). This entry of microbes through the epithelial barrier or the intestine may be facilitated by corticotropin-releasing factor (CRF), a mast cell product, which can increase epithelial permeability and allow access to immune cells in the lamina propria (Rhee et al, 2009). Further evidence from autopsy studies show structural damage to the gastrointestinal tract in schizophrenic patients; 92% had colitis, 88% had enteritis and 50% had gastritis (Hemmings, 2004). These data suggest the GI tract is not fully functional which allows bacterial metabolites to enter circulation which may cause effects on the brain.

The microbiota also modulates neurotrophins (Soto et al, 2018) which are important for brain development and plasticity. Neurotrophins have an important role in regulating development and maintenance of the peripheral and CNS function. Brain-derived neurotropic factor (BDNF) is the most studied neurotrophin in the CNS and in healthy individuals it is correlated with the induction of long-term potentiation (Aicardi et al, 2004). However, BDNF has been shown to be abnormally regulated in the CNS of animal models relevant to schizophrenia (Angelucci et al, 2000). A study in schizophrenic patients showed significantly reduced serum BDNF levels at the onset of psychosis, with greater a reduction of BDNF levels in females (Jindal et al, 2011). BDNF may be responsible for the NMDA-R dysfunction seen in schizophrenia, which contributes to the pathophysiology of the disease including reduced brain plasticity in affected individuals (Angelucci et al, 2005). BDNF regulates the expression of NMDA receptors in cultured hippocampal neurons and increases the mRNA levels for NR1, NR2A and NR2B subunits (Caldeira et al, 2007). Likewise, NMDA-R antagonists have been shown to prevent the action of BDNF (Crozier et al, 1999).

An altered microbiota may contribute to the dysfunctional NMDA-R as it witnessed in schizophrenic individuals through this mechanism. A normal microbiome is required to stimulate brain plasticity by influencing the expression of BDNF and NMDA-R; studies in GF mice have shown decreased levels of BDNF expression in the cortex and hippocampus along with reduced NR2B expression in the NMDA receptors (Leung and Thuret, 2015). Therefore, an abnormal microbiota may be responsible for the dysfunction of these mechanisms which have direct effects on cognition (Maqsood and Stone, 2016).

The intestinal microbiota also plays a role in the innate and adaptive immune responses. The microbiota causes these changes through the accumulation of lymphocytes and differentiation in the GI tract (Artis, 2008). Altered immunity in schizophrenic individuals include abnormal T-cells (Li et al, 2018), increased levels of IL-1β, IL-2, IL-6, and interferon (IFN)-γ (Lesh et al, 2018), increased inflammatory cytokines (Boerrigter et al, 2017), increased chemokines (Hong-Li et al, 2017), and increased C-reactive protein (CRP) (Joseph et al, 2015). For example, altered levels of cytokines within systemic circulation can directly affect the function of the brain; they impact neurocircuits which include the basal ganglia and anterior cingulate cortex, which causes changes in motor activity, anxiety and alarm (Miller et al, 2013). Cytokine levels are also correlated with the severity of schizophrenia symptoms and the resulting neuroinflammation could contribute to its pathogenesis (Rogers et al, 2016).

10% of the brain consists of microglia which is an immune component; they release cytokine and are involved in first line innate immunity of the CNS (Yang et al, 2010). In schizophrenia, microglia activation has been found in the hippocampal area where immediate memory and emotional integration is impaired (Beumer et al, 2012) causing changes in cognition and behaviour. Studies have suggested there is a physiological role for cytokines in memory and learning through the signalling cascades and transcription factors MAPK and STAT which activate cytokines during an inflammatory event (Donzis and Tronson, 2015). Epigenetic modifications and neurogenesis may mediate the long lasting effects of inflammation on memory and cognitive function (Donzis and Tronson, 2015). Production of the cytokines TNFα and type II IFNγ are associated with tryptophan degradation to tryptophol and palmitoleic acid metabolism by gut bacteria (Schirmer et al, 2017) suggesting an altered microbiome composition alters plasma cytokine levels (Lin et al, 2019). Cytokines also increase oxidative stress in tissues by raising the concentration of

nitric oxide (Rosenkranz et al, 1994) and by activating the hypothalamic–pituitary–adrenal (HPA) axis (Dunn, 2000). This leads to the release of the stress hormone, cortisol, and could contribute to the positive and negative symptoms of schizophrenia (Walker et al, 2013). Therefore, it is likely that the enteric microbiota affects the behaviour witnessed in schizophrenia by causing changes to circulating and brain cytokine concentrations.

The vagus nerve is important for communication between the gut and the brain. IL-6 can bind to receptors on the vagus nerve which signals to the hypothalamic brain nuclei via the brainstem (Johnston and Webster, 2009). The cytokine signal gets amplified in the CNS which activates microglia who then secrete proinflammatory cytokines and chemokines within the brain (Ramesh et al, 2013). This activates the enzyme Indoleamine-pyrrole 2,3-dioxygenase (IDO1) which metabolises tryptophan which is involved in serotonergic and dopaminergic transmission. Studies which have colonised GF mice show an increase in tryptophan metabolism (Gao et al, 1018), which leads to more than double of concentrations of 5-HT and its metabolites, which include kynurenic acid, in the brain. Interestingly, increased cytokines and kynurenic acid levels in cerebrospinal fluid (CSF) have been reported in patients with schizophrenia (Kegel et al, 2017).

Specific changes to the gut architecture in PND's in currently lacking. However, there are numerous comorbidities which have been reported between psychiatric illness and GI disorders such as IBS (Lee et al, 2015), IBD (Bernstein et al, 2019) and celiac disease (Carta et al, 2015), suggesting an impact of the gut on the brain. Most evidence indicates that the tight junction integrity of the epithelial layer of the gut is compromised. Genes such as claudin-5 (Campbell, 2019) and other molecules such as nitric oxide synthetase (Chokshi et al, 2008) are both involved in epithelial barrier integrity and have been identified as susceptibility loci in schizophrenia cases (Severence et al, 2015). Gut epithelial barrier integrity is also heavily influenced by inflammation (Pastorelli et al, 2013), infections (Ziegler et al, 1998) and stress (Kelly et al, 2015). Stress is a risk factor for worsening the symptoms of schizophrenia (Walker et al, 1997). Commensal microbes maintain epithelial barrier integrity by downregulating inflammatory responses and expressing anti-microbial proteins (Menard et al, 2004), and repairing damaged intestinal tissue (Coleman and Haller, 2017).

Specific bacteria have roles including the maintenance of cadherin desmosomes (by inducing the expression of proline-rich protein 2A) by *Bacteroides thetaiotaomicron* (Tungland, 2018); stabilisation of tight junctions by several *Lactobacillus* strains (Yang et al, 2015); correction of gut permeability is aided by *Bacteroides fragilis* (Deng et al, 2018); and bioactive factors secreted from *Bifidobacterium infantis* enhance epithelial barrier function (Ewaschuk et al, 2008). As mentioned previously, schizophrenia patients have significantly fewer *Bifidobacterium* and *Lactobacillus species* (Cuomo et al, 2018). This suggests that an altered microbiome impairs the integrity of the epithelial barrier which allows bacterial translocation and could potentially increase the risk of developing PND's including schizophrenia.

However, although there have been correlational studies between the microbiota and PNDs, clinical data can be compromised by the high use of antibiotics and dietary variations in affected individuals. GF studies in mice have shown social deficits and increased repetitive behaviours like those in autism, suggesting the microbiota may have a role in the development of some symptoms of ASD (Borre et al, 2014). Few clinical studies have shown any beneficial effects for probiotic supplementation in schizophrenic patients or with other PNDs (Dickerson et al, 2014). One study which used a combined *Lactobacillus rhamnosus* and *Bifidobacterium animalis* probiotic supplementation found no significant effects on the positive and negative symptoms of schizophrenia; there were only improvements to general gut health (Dickerson et al, 2014). Another study found that probiotics had limited efficacy in the management of behaviour symptoms in the children with ASD (Ng et al, 2019).

1.7 Mouse models of Schizophrenia

Mouse models are commonly used to analyse the gut microbiota and other investigations due to their similarities in anatomy, physiology and genetics as humans. They also have relatively low maintenance costs, high reproductive rates and a short life cycle. GF models allow interventions which cannot be performed on humans, and provide key evidence on how gut bacteria has an influence on the metabolism of the host (Bäckhed et al, 2007). Mouse models are useful because they allow perturbations in the gut microbiota to be studies in a controlled environment where factors such as diet and housing conditions can be manipulated to prevent unwanted influences on the gut microbiota from the surrounding environment.

Both mice and humans have GI tracts which are anatomically similar. They have similar sectional tissues and cells which include goblet cells, EECs, absorptive enterocytes and Paneth cells (in the small intestine), and absorptive colonocytes, goblet cells, EECs, microfold cells (in the colon). Furthermore, the ratio of intestinal surface area to body surface area is similar between both mice and humans (Casteleyn et al., 2010). Mouse colonic mucous consists of two layers with similar protein compositions, and MUC2 is the major structural component. Mucins are part of innate immunity and are well conserved. MUC2 is a larger glycoprotein characterised by O-linked glycans attached to hydroxy amino acids clustered in mucin domains (Johanson et al, 2008). The outer mucus layer is colonised with bacteria and but is separated from the epithelium (Johanson et al, 2008).

Although mice and humans share many similarities with regards to their GI tract, there are also some differences which should be considered. The small intestine in mice has taller villi with no mucosal folds whereas humans have shorter villi with mucosal folds to increase the surface area (Smith et al, 2013). Transverse folds in humans are present along the length of the colonic mucosa, however in mice they are restricted to the proximal colon and caecum (Ngugen et al, 2015). The colon of humans is more complex; it is sectional which includes ascending, descending and transverse areas whereas mice have a more simplified colon. Paneth cells, which are key effectors of innate mucosal defence, are present in the caecum and proximal colon of humans but only reside in the small intestine of mice (Cunliffe et al, 2001). Additionally, goblet cells which secrete the important mucin, MUC2, are abundant from the colon through to the rectum in humans but are only

abundant in the proximal colon of mice (Ngugen et al, 2015) ; there abundance decreases at the base of the crypts in the distal colon and in the rectum. Mice also have a functional caecum for fermentation; humans do have a caecum although it is smaller and not functional. The caecum in mice is still under investigation with regards to gut microbiota research as it resembles the function of the human appendix. The human appendix is likely a remnant of the caecum which was derived from dietary selection pressures (Smith et al, 2013), and research suggests that it has the ability to replenish gut microbiota following disturbances (Smith et al, 2013).

Genera with high abundance in human gut microbiota, as compared to mouse gut microbiota, include *Prevotella*, *Faecalibacterium* and *Ruminococcus*, whereas *Lactobacillus*, *Alistipes* and *Turicibacter* are more abundant in mouse gut microbiota. *Clostridium*, *Bacteroides* and *Blautia* have a similar relative abundance in both organisms (Wang et al, 2019).

Like humans, different mice models will have shifts within their microbiota compositions, however this is to be expected. The crosstalk between the microbiota and host is host-specific so the observations concluded within mice models is possibly not applicable in humans. Furthermore, inbred strains of mice will not represent the genetic variation which is present in the human population. Finally, important factors such as type of birth, diet, genetic background, medical history contribute to a health gut microbiota in humans but this lacks in mouse models so their gut microbiota may not be as representative as it could be. In summary, mice models do not fully represent the human system however they are ideal for research investigations due to their appropriate similarities.

1.7.1 Phencyclidine (PCP) mouse model of Schizophrenia

Phencyclidine (PCP) is a non-competitive NMDA-R antagonist which causes NMDA-R hypofunction. Heterotetrametric NMDA-R's are widely distributed throughout most of the brain and have an important role in mediating synaptic plasticity. Their presence has also been demonstrated in the enteric nervous system (Zhou and Verne, 2008). Meta-analysis has shown significant decreases in the expression of glutamate ionotropic receptor NMDA type subunit 1 (GluN1) mRNA and GluN1 protein in the prefrontal cortex (PFC) of subjects with schizophrenia (Catts et al, 2015). In mouse models, NMDA-R antagonists such as PCP causes neurochemical and cognitive defects which are similar to

those observed in schizophrenia (Meltzer et al, 2013). It has been proposed that the cognitive defects are due to hypofunctional NMDA-Rs on cortical GABA interneurons which changes cortical network oscillations (Balu, 2016).

Previous studies in mice using PCP have found the drug to cause changes in behaviour relevant to schizophrenia. These include elevated motor activity and impaired novel object recognition when PCP was repeatedly administrated for 7 days (Nomura et al, 2016, Young et al, 2012). Other studies in mice have also shown evidence of partial social withdrawal (Brigman et al, 2009).

PCP may induce these behaviours by causing a long-lasting metaplastic change in the inhibitory circuits in the hippocampus that results in impaired long-term potentiation of CA1 excitatory synapses; these correlate with an increase in inhibitory GABA input to CA1 pyramidal neurons (Nomura et al, 2016). Other studies have shown that the effects of repeated PCP administration decrease the density of 5-HT receptors in mice hippocampus (Hagiwara et al, 2008). These effects can be reversed by antipsychotic drug treatment, for example treatment with perosprione for 14 days attenuated the cognitive deficits caused by PCP via 5-HT receptors (Hagiwara et al, 2008).

Although there is vast proven evidence that PCP induces psychotic effects, little work has been done to explore the possibility of the involvement of the gut microbiota and the possible link between the gut composition and brain function. However, a study by Jørgensen et al, 2015, showed that administration of sub-chronic PCP for 3 weeks triggered a stress response which affected the function of the GI tract, mucus secretion and motility, and in turn altered the composition of the gut microbiota. Their findings also showed impaired novel object recognition after washout, and increased locomotor sensitivity which continued for up to 6 weeks after washout. Their gut microbiome had also been altered which suggests sub-chronic PCP treatment has a potential role for the gut microbiome in these alterations, however the link between the two has not yet been proved and requires far more investigation and proof. These data suggest that the microbiota potentially has a role in the PCP model of schizophrenia.

As PCP is an NMDA-R antagonist and these receptors are present in the enteric nervous system, we would expect PCP to bind here and potentially cause disturbances to the gut architecture and microbiome. Studies in mouse models sub-chronically treated with PCP to investigate the gut microbiota with regards to cognitive function are severely lacking and more studies need to be carried out to provide more evidence of the role of the gut microbiota in diseases such as schizophrenia.

1.7.2 Neurexin1 gene knockouts

Another mouse model now being utilised in the context of preclinical schizophrenia research are *Nrxn1* gene knockouts. As mentioned earlier, the Nrxns 1-3 are a group of synaptic adhesion proteins involved in the formation and maintenance of synapses (Wright and Washbourne, 2011). *Nrxns* are cell-surface receptors that bind neuroligins to form Ca^{2+} - dependent neurexin/neuroligin complexes at synapses in the CNS which is vital for neurotransmission (Reissner et al, 2013). The *Nrxn1* gene produces *Nrxn1α* protein which is vital for synaptic adhesion and function. They also induce post-synaptic differentiation in glutaminergic synapses and regulate NMDA-R function (Graf et al, 2004). The *Nrxn1α* extracellular domain consists of 6 laminin-neurexin-sex hormone binding globulin domains (LNS domains) with 3 epidermal growth factor (EGF)-like repeats and bind with ligands such as neuroglins, neurexophilin, α-dystroglycan and GABA-receptors (Chen et al, 2011). In addition to the CNS and highly enriched synaptic regions of the ventral ganglion and brain, there is evidence that *Nrxn* and neuroglins are also expressed in the enteric nervous system in the gut (Wang et al 2017).

In mouse models of *Nrxn1* heterozygous deletions, behavioural changes include no social memory for familiar versus novel objects and lack of long-term object discrimination when compared to wild-type controls (Dachtler et al, 2015). Other observed behaviours include increased grooming behaviours and impairments to nestbuilding activity (Etherton et al, 2009). These behaviours in mice are considered relevant phenotypes for comparison to schizophrenia and autism in humans because they are suggestive of OCD which is present in many schizophrenia cases (Schirmbeck and Zink, 2013). These behaviours were likely caused by defects in excitatory synaptic strength (Sudhof, 2017), as has been shown by decreases in miniature excitatory postsynaptic current (EPSC) frequency (Etherton et al, 2009). Decreases in prepulse inhibition have also been observed (Etherton et al, 2009).

In addition to schizophrenia, *Nrxn1* gene (2p16.3) deletions have been linked to an increased likelihood of developing other neurodevelopmental disorders including ASD (Dachtler et al, 2015), attention deficit hyperactivity disorder (ADHD) (Lowther et al, 2015), and Tourette's syndrome (Fernandez et al, 2012). Although several studies show significant associations between *Nrxn1α* deletions and the susceptibility to PND'S, so far little has been done to investigate an association with the microbiome.

1.8 The importance of GLP-1 in gut function and the potential role of GLP-1 receptor agonists in schizophrenia

GLP-1 is an incretin hormone produce by EEC's (L-cells) in the distal ileum and colon of the gut and increases glucose-stimulated insulin secretion in the pancreas through the regulation of ion channels (Macdonald et al, 2002). It's production and release is upregulated by nutrients including glucose and fatty acids (Bodnaruc et al, 2016) which increase the transcription of the glucagon (GCG) gene which encodes GLP-1 (Doyle and Egan, 2007). The actions of GLP-1 on target tissues, such as the GI tract and CNS (Cabou and Bercelin, 2011), is mediated by a single G-protein-coupled receptor isoform (Macdonald et al, 2002). In the gut, GLP-1 is known to affect gut motility, inhibit gastric acid secretion, and inhibit glucagon secretion by acting as an incretin (Macdonald et al, 2002). Current research developments suggest that GLP-1 may have other effects besides insulin secretion, such as reducing inflammation and neuroprotection (Lee and Jun, 2016).

GLP-1 agonists such as Liraglutide have a potential role in the improvement of schizophrenia symptoms. Liraglutide is a modified analogue of GLP-1, with an arginine to lysine amino acid substitution at position 34 and the attachment of a glutamic acid residue with palmitic acid to an existing lysine residue at position 26 (Wajcberg and Amarah, 2010). Liraglutide shares 97% homology with human GLP-1 and is resistant to cleavage by dipeptidyl peptidase 4 inhibitor (DPP-IV) (Gupta, 2013). Liraglutide has a greater half-life (13 hours) (Sisson, 2013) than GLP-1 and is able to readily cross the BBB (Candeias et al, 2015). Studies in mice have shown GLP-1 agonists such as Liraglutide to exert antipsychotic-like effects in mice (Dixit et al, 2013). Other studies have shown GLP-1 analogues to improve learning and memory dysfunction, possibly by enhancing the regulation of glucose in the brain (Bae and Song, 2007). GLP-1 agonists may also prevent metabolic and cerebral deficiencies in schizophrenia patients with antipsychotic-induced weight gain (Ebdrup et al, 2012).

There is a high rate of comorbidity between schizophrenia and type II diabetes (Suvissari, 2008). In previous studies in our lab we have shown that the GLP-1 receptor agonist Liraglutide can improve cognition and brain function in mice heterozygous for *Nrxn1α*

(unpublished observations), therefore here we will test the effects of Liraglutide on the gut architecture and microbiome.

1.9 Aims of the project

In this project we will be analysing changes, to the gut architecture in mice colon and caecum in two mouse models relevant to schizophrenia. Firstly, in mice treated with PCP, and secondly, in mice heterozygous for *Nrxn1α*. To determine the impact of Liraglutide on gut function and the microbiome we will analyse effects in animals treated with both saline and Liraglutide. In terms of gut architecture, we will characterise the colon and crypt lengths, abundance and size of mucus secreting goblet cells, and the thickness of the muscle layer. The abundance and phyla of bacteria in the gut will also be investigated between the sample groups because targeting the microbiome may offer a novel strategy to improve brain function in these models, and so in schizophrenia. The novel data will provide the first evidence that genetic risk factors for these disorders impact on the microbiome and will also identify the specific bacterial populations affected.

2. Materials and Methods

2.1 Materials

Reagent	Supplier	Catalogue number
Poly-L-lysine	Sigma-Aldrich	P8920
Periodic Acid	Sigma-Aldrich	395132
Schiff's reagent	Sigma-Aldrich	S5133
Alcian blue (AB)	Sigma-Aldrich	B8438
Haematoxylin	Sigma-Aldrich	Hhs16
Anti-CgA primary antibody	Abcam	ab15160
Anti-GLP-1 primary antibody	Invitrogen	Pas-79303
488 donkey anti-rabbit IgG secondary antibody	Invitrogen	A-11055
Donkey serum	Sigma-Aldrich	D9663
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A3294
Sybr gold	Invitrogen	S11494
Triton X-100	Sigma-Aldrich	X100PC
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	T9281
Dibutylphthalate Polystyrene Xylene (DPX)	Sigma-Aldrich	06522
341 6CF forward primer	5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGCSCGGGGGGCCTACGGGSGGCSGCSG-3'	
518 R reverse primer	5'-ATTACC GCG GCT GCT GG -3'	
4',6-diamidino-2-phenylindole (DAPI)	New England Biolabs	
Vectashield	Vector Laboratories	H1000
Ammonium persulphate (APS)	Sigma-Aldrich	A3678
QIAamp fast stool kit (Proteinase K, Inhibitex buffer, Buffer AL, Buffer AW1, Buffer AW2, Buffer ATE)	Qiagen	51604
QIAquick PCR purification kit (Buffer PB, Buffer PE, Buffer EB)	Qiagen	24104
Acrylamide	Fisher	BP1406-1
Formamide	Sigma-Aldrich	F9037

Table 2: Table of materials used in this study. Table shows name of reagent, supplier information, and catalogue number.

2.2 Methods

2.2.1 Animals

In our first study we used mice subchronically treated with PCP, where "subchronic" refers to PCP exposure for a moderate duration of time, and in our second study we used mice with a heterozygous gene deletion of *Nrxn1*α (see below). The mice in our experiments were group housed with 4-6 animals per cage under standard conditions, where cages were ventilated individually at 21°C at 45-65% humidity. We utilised a dark/light cycle where lighting conditions changed every 12 hours (lights on at 6:00am). Our mice had access to food and water *ad libitum*. Our experiments were carried out in compliance with the UK animals (Scientific procedures) Act 1986 and approved by Lancaster University Animal Welfare and Ethics Review Board.

The mice in our experiments were aged 8-12 weeks when gut tissue samples from the colon and caecum were taken.

(1) Mice treated sub-chronically with PCP (10mg/kg administered intraperitoneally (i.p.), once a day for 5 days and samples collected 72 hours after the final drug administration). Controls received saline injection (2mls/kg). These doses have worked efficiently in previous studies (Hashimoto et al, 2007; Santini et al, 2013), hence why we replicated them in our study. PCP females n = 4, PCP males n = 4, saline females n = 4, saline males n = 4.

(2) *Nrxn1*α heterozygous mice were generated from male mice who had a heterozygous knockout at *Nrxn1*, homozygous knockout at *Nrxn2* and wild-type at *Nrxn3*. These mice were outbred once to a strain of C57BL/6NCrl mice to obtain mice that were individually either *Nrxn1*α or *Nrxn2*α knockout heterozygotes (Dachtler et al, 2015). We used *Nrxn1*α heterozygous mice in our study. Our *Nrxn1*$\alpha^{+/-}$ and wild-type mice were treated with saline or Liraglutide. *Nrxn1*$\alpha^{+/-}$ saline females n = 6, *Nrxn1*$\alpha^{+/-}$ Liraglutide females n = 7, *Nrxn1*$\alpha^{+/-}$ saline males n = 5, *Nrxn1*$\alpha^{+/-}$ Liraglutide males n = 5, wild-type saline females n = 6, wild-type Liraglutide females n = 6, wild-type saline males n = 4, wild-type Liraglutide males n = 4.

2.2.2 Coating Slides with Poly-L-Lysine

Untreated microscope slides were submerged in a solution of hydrochloric acid (HCL) in 70% ethanol for 5 minutes (350ml 100% ethanol, 13.7ml 1M HCL, and 136.3ml dH20). Slides were then washed 2 times in distilled H_20 (dH_2O), for 2 minutes 30 seconds each. Finally, slides were submerged in a 1:10 dilution of poly-L-lysine (30ml poly-L-lysine (0.1% w/v solution, Sigma-Aldrich, UK) and 270mL dH_2O). Poly-L-lysine must be handled using plastic laboratory equipment only. Slides were covered with paper towels to reduce dust contact and are air-dried overnight. They were then stored at 4^oC in foil until use.

2.2.3 Tissue Processing and Sectioning

1. Tissue Processing

Mouse colon and caecum tissue samples, stored inside histological cassettes (Sigma-Aldrich, UK), were fixed in Carnoy's solution (made from methanol, chloroform and acetic acid) for 4 hours, preserving the mucus layer. Fixed tissue was then placed in 100% methanol for 1 hour followed by 100% ethanol for 30 minutes, followed by 3 washes in xylene for 15 minutes each. The tissue was then submerged in two trays of hot paraffin wax for 30 minutes each.

2. Wax Embedding

A layer of hot paraffin wax was dispensed into a $3cm^2$ metal mould. Samples were removed from their histological cassettes using forceps and placed into the mould with the lumen of the samples perpendicular to the bottom face of the mould. The mould was placed on wet ice to promote wax setting and to ensure the sample remained in position. The top layer of the cassette was then replaced, and the mould filled to the top with more hot wax and replaced onto the ice. Once the wax had set and samples have cooled, samples in cassettes were removed from the mould and excess wax was removed.

3. Tissue Sectioning

Embedded samples were placed securely into the microtome. After removing safety guards and setting the cutting thickness to 5µm, samples were sectioned and floated onto a layer of warm (40^{O}C) water to remove creases and folds in the section. Poly-L-lysine coated slides were used to pick up 3 sections per slide. The slides were then left to air dry before staining. Per slide, one section was stained with Alcian blue (AB), one AB/Periodic acid Schiff (PAS) combined, and the other PAS (see below).

2.2.4 Histological Staining and Microscopy

Periodic Acids Schiff's (PAS) and Alcian Blue (AB) Staining

PAS stain is used to stain neutral and acidic non-sulphated mucins and glycoproteins (Shafiei, 2014). AB is a cationic dye used to stain acidic mucins by binding to the anionic sulphur and carboxyl groups within the mucins (Dong et al, 2012). Each section of tissue sample per animal was stained with PAS or AB stain, or a combined PAS/AB stain.

Slides containing tissue sections were first dewaxed in two washes of xylene, for 10 minutes each, in a fume cupboard. Samples were then washed in ethanol (100% ethanol x2 washes for 2 minutes each. Samples were then washed in dH2O (x3 washes for 2 minutes each), and then stained with AB (Sigma-Aldrich) for 5 minutes. After washing again in dH2O (x3 washes for 2 minutes each), samples were stained with 1% periodic acid reagent (Sigma-Aldrich) for 5 minutes under dark conditions to prevent the reaction of the solution with light. After another washing stage in dH_2O (x3 washes for 2 minutes each), samples were stained with Schiff's reagent (Sigma-Aldrich) for 15-30 minutes, and then washed under running tap water for 5 minutes. Samples were then stained with haematoxylin (Sigma-Aldrich) for 1 minute then rinsed in warm water and washed in running warm tap water for 2 minutes. Finally, samples were dehydrated in a series of increasing ethanol washes (70% for 2 minutes, 90% for 1 minute, and 100% x2 for 2 minutes) and were then placed in xylene (x2 washes for 2 minutes each). After samples had air dried DPX (Sigma-Aldrich) mountant was used to seal coverslips over the tissue samples.

2.2.5 Characterisation of Gut Architecture and EEC numbers

2.2.5.1 Gut Architecture Analysis - Light Microscopy

Gut architecture was analysed microscopically under the light microscope at x10 and x40 magnifications. Samples stained with PAS, AB, or PAS/AB combined were photographed and subject to analysis using ImageJ software (NIH, https://imagej.nih.gov/ij/docs/intro.html). We calibrated our measurements by taking an image of an accurate scale bar under the microscope and then we used ImageJ to take accurate measurements with the line drawing tool. 20 random crypts were analysed per

sample, with crypt length (µm), number and size of goblet cells (μm^2), and muscle layer depth (µm) recorded.

2.2.5.2 EEC Numbers - Fluorescence Immunohistochemistry

Fluorescent immunohistochemistry was carried out using a primary antibody raised against CgA (anti-CgA, Abcam) or GLP-1 (anti-GLP-1, Invitrogen) conjugated to 488 donkey anti-rabbit IgG secondary antibody (Invitrogen) to assess EEC numbers in the gut. CgA stains all EEC's present, whereas GLP-1 antibody only stains a subset of EEC that are positive for the protein. Sections were deparaffinised by 2 washes in xylene for 10 minutes each and then rehydrated in decreasing ethanol gradients of: 2 washes in 100% ethanol, 1 wash in 90% and then one wash in 70% ethanol, each for 2 minutes. The sections were then washed three times in 1 x (full name) Phosphate-buffered saline (PBS) buffer (Made from 800mL dH_20, 8g NaCl, 200mg of KCl, 1.44g Na_2HPO_4, and 240 mg of KH_2PO_4) for 2 minutes.

Antigen retrieval was carried out to remove covalent crosslinks formed during the fixation process that may have blocked antibody binding sites. To achieve this the sections were immersed in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and heated at full power in a microwave oven for 10 minutes. When cool, the sections were then washed in 1x PBS for 1 min. A Peroxidase-Antiperoxidase (PAP) pen was used to create a hydrophobic barrier around each tissue section.

The tissue cells were permeabilised by incubating with 0.25% Triton X-100 in 1x PBS for 10 minutes. A blocking step was carried out to block any non-specific antigens by incubating for 30 minutes with 10% donkey serum in 1x PBS containing 3% BSA. The sections were then incubated for 18 hours at 4°C with the anti-CgA or anti-GLP-1 primary antibody, both diluted 1:200 in 1x PBS containing 3% BSA.

Following incubation, the sections were washed three times in 1x PBS for 2 minutes. All subsequent steps were carried out in the dark at room temperature. The secondary 488 donkey anti-rabbit IgG (H+L) antibody (Invitrogen), diluted 1:500 in 1x PBS, was incubated on the sections for 1 hour. The sections were then washed three times in 1x PBS for 2 minutes each. 4',6-diamidino-2-phenylindole (DAPI) (New England Biolabs), diluted 1:1000 in 1x PBS, was incubated on the sections for 5 minutes. The slides were mounted with

Vectashield (Vector Laboratories) and a coverslip fixed in place. The sections were stored in the dark at room temperature until required and viewed using the Zeiss Axio Scope A1 microscope.

EEC numbers, stained positive for CgA or GLP-1, were counted visually for each sample, with the total number of fluorescent cells per 20 crypts recorded. Image J software was used for fluorescent analysis.

2.2.6 Analysis of the Gut Microbiome

2.2.6.1 16S DNA Extraction and Purification

Bacterial 16S DNA extraction was carried out using the QIAGEN QIAmp Fast DNA Stool Mini Kit (catalogue number: 51604) in accordance with the manufacturer's instructions, with all centrifugation steps performed at 20,000g. Each colon and caecum sample was retrieved from the -80^{O}C freezer and defrosted on ice. Scissors were washed in disinfectant and ethanol before contact and in-between contact of the different samples to prevent cross-contamination. Colon and caecum samples were dissected to expose the faecal contents of the lumen. 1mL of Inhibitex buffer was added to each stool sample and a sterile inoculation loop used to break down the stool. Faecal content and Inhibitex buffer were then pipetted into a 1.5ml Eppendorf tube and vortexed vigorously until the stool sample was fully homogenised. The suspension was then heated for 5 minutes at 95^{O}C and centrifuged for 1 minute to pellet the stool particles. 30uL of Proteinase K and 400µl of the supernatant was added to a new 1.5mL microcentrifuge tube, along with 400µl buffer AL from the kit, and the samples mixed by vortexing. The samples were then left to incubate at 70^{O}C for 10 minutes.

400µl 100% ethanol was then added to each sample lysate and is mixed by vortexing. 600µl of each lysate was then added to a spin column and centrifuged for 1 minute, and this process repeated until all of the lysate was been added to the spin column. The QIAmp spin column was then placed in a new 2ml collection tube, and the tube containing the filtrate is discarded. 500µl of buffer AW1 was then added to the spin column, centrifuged for 1 minute and the filtrate discarded. 500µl buffer AW2 was then added to the spin column and

centrifuged for 3 minutes, transferred to a new collection tube and centrifuged for a further 3 minutes. Finally, the QIAmp spin column was transferred to a new 1.5ml microcentrifuge tube (with the lid removed). 100µl buffer ATE was pipetted directly onto the QIAmp spin column membrane and was left to incubate at room temperature for 5 minutes. All samples were then centrifuged for 1 minute to elute the DNA.

2.2.6.2 Quantification of total 16S DNA

A nanodrop spectrophotometer was used to quantify the DNA concentration (ng/ml) in the sample. The measuring device was calibrated using ATE buffer as the blank control to avoid false readings.

2.2.6.3 Amplification of 16S DNA

DNA concentrations were diluted to make up a concentration of 100ng DNA in 9.5µl of nuclease free water. The 341 6CF forward primer and 518R reverse primer (see Table 1 for sequence information) we used were at 10µM concentration and diluted to 1:10. A mastermix containing 12.5µl RedTaq mastermix (Sigma-Aldrich), 1µl 341 6CF forward primer, 1µl 518 R reverse primer, 1µl BSA (Sigma-Aldrich), and 9.5µl nuclease free water per sample, with 100ng of each DNA sample in 9.5µl of nuclease free water being added to each PCR tube. Samples were vortexed with the PCR vortexer and placed into PCR machine with cycling conditions as follows:

1. 95^{O}C for 5 minutes
2. 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute (30 cycles repeated)
3. 72°C 10 minutes

2.2.6.4 Purification of 16S PCR Products

Post-PCR purification was carried out using the QIAGEN MinElute PCR purification kit (catalogue number: 24104) in accordance with the manufacturer's instructions, with all centrifugation steps carried out at 16,000g. 5 volumes of buffer PB were added to 1 volume

of the PCR reaction and mixed. The colour of the mixture needs to be yellow to indicate the correct pH; if orange or violet in colour then 10µl 3M sodium acetate at pH 5 can be added and mixed until it turns yellow.

Samples were placed into each MinElute column within a 2ml collection tube and centrifuged for 1 minute. The flow-through from the collection tube was discarded and the same collection tube was re-used with the same MinElute column. 750µl of buffer PE was added to the MinElute column and the sample again centrifuged for 1 minute. The flow-through is discarded and the collection tube placed back onto the column and centrifuged for 1 minute.

Each MinElute column was then placed into a clean 1.5ml microcentrifuge tube. To elute the DNA, 20µl of buffer EB (10mM Tris CL, PH8.5) was dispensed directly into the centre of the membrane for complete elution of bound DNA. The column was left to stand at room temperature for 1 minute and then centrifuged for 2 minutes to elute DNA. DNA concentration was quantified using nanodrop and samples are stored at -20^{O}C until required.

2.2.6.5 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE requires 150ng of DNA in 16µl of nuclease free water. 100% and 0% denaturant solutions were prepared. 100% denaturant (8.4g Urea, 5ml Acrylamide, 8ml Formamide, and 0.4ml Tris-acetate-EDTA (TAE) (x50) (made from Tris, EDTA, and acetic acid) was mixed using a heated stirrer at 50^{O}C until the urea had been fully dissolved. 0% denaturant (5ml Acrylamide, 14.6ml water and 0.4ml TAE(x50)). 10% APS was also prepared by adding 0.1g to 1ml of distilled water.

A high gradient solution (70%) was prepared by mixing 16.8ml of the 100% denaturant solution and 7.2ml of the 0% denaturant solution. A low gradient solution (30%) was prepared by mixing 7.2ml of the 100% denaturant solution and 16.8ml of the 0% denaturant solution. Both solutions were then chilled in a -20^{O}C freezer for 30 minutes. 225µl of APS

and 12.75µl TEMED (Sigma-Aldrich) were also added to each solution after being taken out of the freezer, immediately before pouring the gel.

Two glass plates at 16cm and 10cm were cleaned using acetone and left to dry. Spinkote lubricant was used to coat two plastic spacers which we attached to the glass plates along the edges. We ensured there was a tight seal between the plates to prevent the gel leaking. The air-tight glass plates were inserted into casting blocks and tightened. We inserted a 21g needle onto the rear end of the tube from the peristaltic pump and placed between the sheets of glass.

2.2.6.6 Pouring the DGGE Gel

The gradient pourer was first cleaned by flushing water through prior to pouring the gel. With the valves closed, the 30% denaturing solution was added on the left side and allowed to pour through to the right side. We replaced the solution on the right with a stripette and re-poured back into the left side. Ensuring all valves were closed, we added the 70% solution to the right side. Then we opened all valves and turned on the gradient pourer, ready for the solutions to flow via peristalsis through the tube and in between the glass plates. A magnetic flea must be present within the 70% solution to prevent the gel from setting too early, and a stripette was used to manually stir the 30% solution gradually. Once the casting plates had reached maximum capacity with the gel solution, we removed the needle and inserted a well comb, then sealed with 8.5ul of TEMED. The gel took 30 minutes to set. When finished with the gradient pourer it was flushed with water to clean.

2.2.6.7 Preparing the Tank and Gel for Loading

The DGGE tank was switched on, allowing enough time to warm to 60°C. 1xTAE buffer must reach the fill line on the tank for heating to proceed. The comb was removed from the set gel and wells flushed with 1xTAE buffer using a 25g needle to remove excess TEMED. The casting block containing the gel within the glass was removed from the casting frame and was inserted into the DGGE tank. DNA samples at 16µl volume were prepared using 150ng of DNA, 4µl of loading dye, and dH20 to make up 16µl. Samples were loaded into the wells of the gel and run overnight at 63V.

2.2.6.8 Staining gel and visualising

The casting block was removed from the DGGE tank and the smaller glass plate removed from the gel. The gel was washed with dH_20 followed by 4µl Invitrogen gold in 20ml dH_20, ensuring full coverage of the gel. Using foil, the gel was covered and left to incubate at room temperature for 30 minutes. The gel was then washed again with dH_20, being careful to handle the gel minimally. We then visualised the gel using UV gel Chemidoc. An inverted image showed the required detail needed for analysis using Phoretix software.

2.2.7 Statistical Analysis

Statistical tests were used to analyse our data and indicate any significance differences between experimental groups. For the majority of the data we used analysis of variance (ANOVA), which tested the differences between the means of our data. We used ANOVA statistical analysis for all gut architecture analysis and gut microbiome DGGE band analysis, with the exception of GLP-1 analysis in *Nrxn1*$\alpha^{+/-}$ mice, where we used Wilcoxon signed-rank test. For the analysis of differences within a group, for example between males and females, we used Tukey's Honest Significant Difference testing which is a post-hoc test used to find means that are significantly different from each other. For gut microbiome clustering analysis, we used the Adonis technique from the vegan package in the R programme, which is based on the permutation of distance matrices.

3. Results

3.1 Gut Architecture

3.1.1 Impact of subchronic PCP administration

To examine the features of the gut histologically, 4µm sections of colon and caecum were stained with PAS/AB stain and examined microscopically. Colon and caecum crypt length, the size and abundance of goblet cells, and the depth of the muscle layer were compared in mice treated with either PCP or saline. EECs were examined using DAPI and either the CgA or GLP-1 antibody to assess their abundance.

3.1.1.1 Muscle layer depth is thicker in PCP-treated mice

In the colon, mice treated subchronically with PCP had a significantly thicker muscle layer as compared to saline controls ($p = 0.012$, $F(1,10) = 9.366$, Figure 3a). There was no significant evidence that sex influenced the muscle layer depth in the colon ($p = 0.666$, $F(1,10) = 0.202$, Figure 3b). By contrast, in the caecum there was no significant difference in muscle layer depth between PCP and saline-treated mice ($p=0.366$, $F(1,12) = 0.882$, Figure 3c). However, the caecum muscle layer depth was significantly thicker in females as compared to males ($p = 0.003$, $F(1,12) =13.949$, Figure 3d). We also observed no sex x treatment interactions in the colon ($p = 0.518$, $F(1,10) = 0.449$) or in the caecum ($p = 0.896$, $F(1,12) = 0.018$). Representative PAS/AB stained images taken at x40 magnification are shown in Figure 4 and Figure 5.

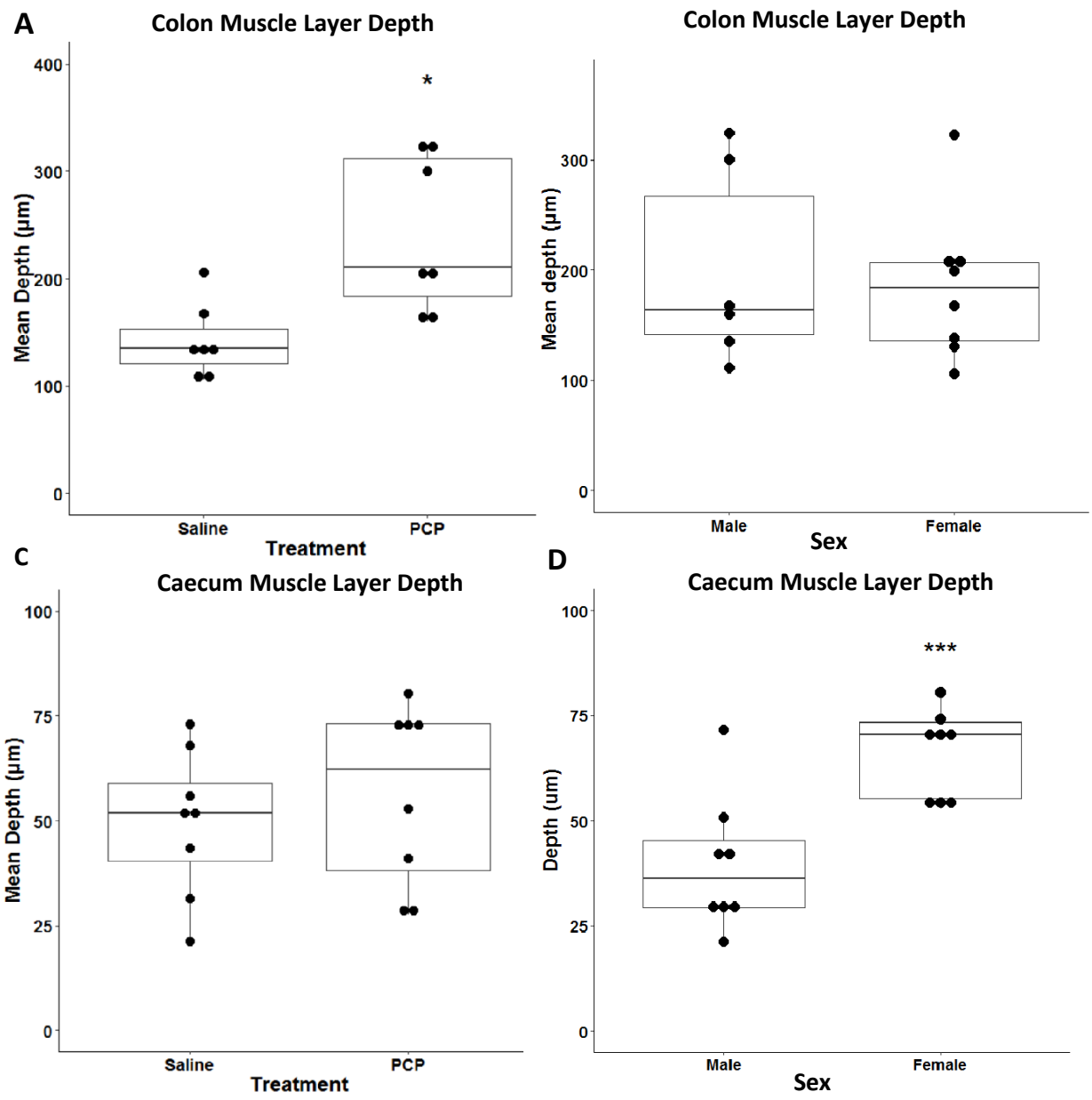


Figure 3: Subchronic PCP treatment increases muscle layer depth in the colon but not in the caecum. (a) Colon muscle layer depth (µm) in saline and PCP-treated mice (saline n = 8, PCP n = 8, p = 0.012, $F_{(1,10)}$ = 9.366). (b) Colon muscle layer depth in female and male mice (females n = 8, males n = 8, p = 0.666, $F_{(1,10)}$ = 0.202). (c) Caecum muscle layer depth (µm) in saline and PCP-treated mice (saline n = 8, PCP n = 8, p = 0.36615, $F_{(1,12)}$ = 0.882). (d) Caecum muscle layer depth in female and male mice (females n = 8, males n = 8, p = 0.003, $F_{(1,12)}$ = 13.949). *P<0.05 and ***P<0.001 compared to relevant control (ANOVA).

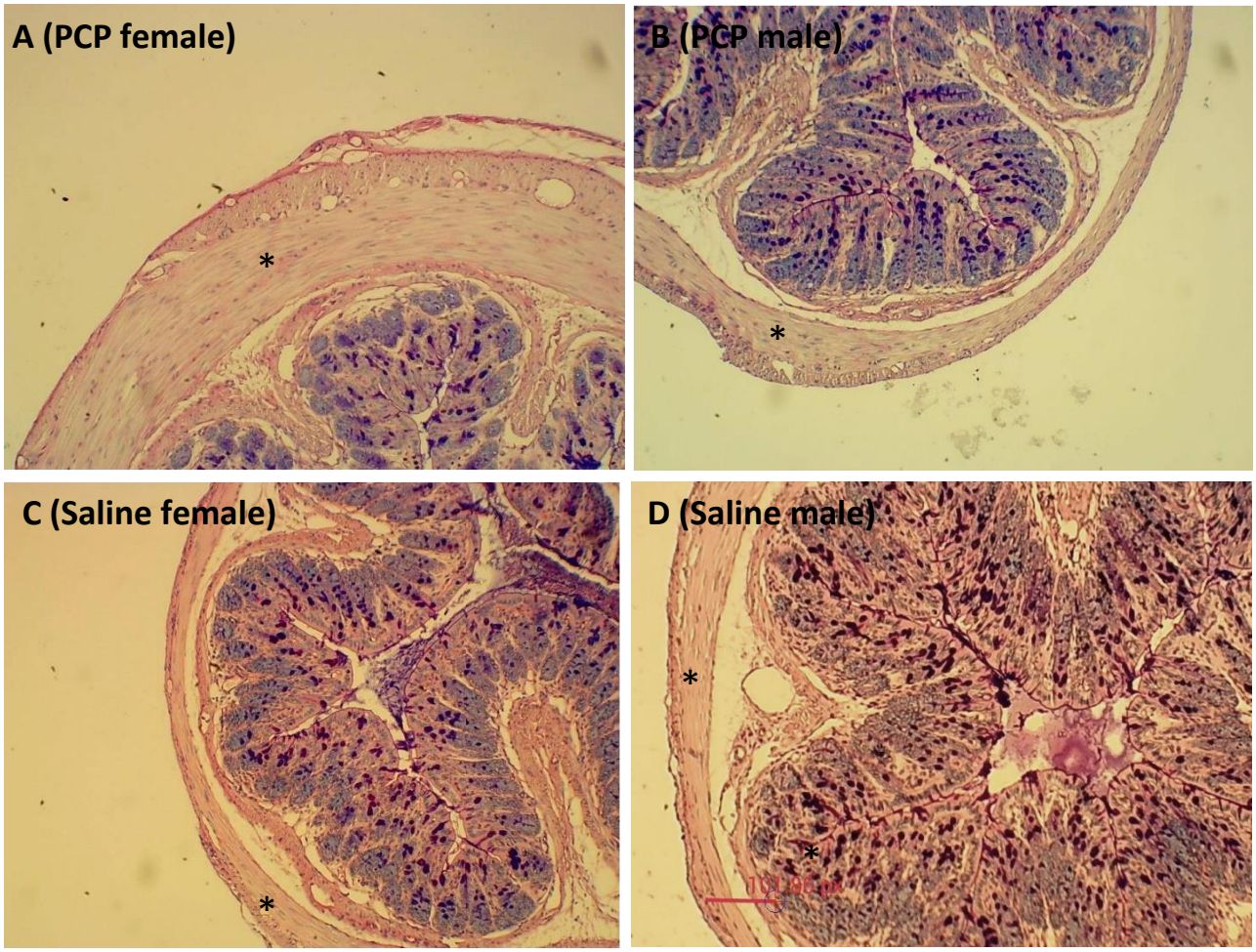


Figure 4: Representative PAS/AB stained colon sections. Muscle layer sections from (a) PCP-treated female mouse, (b) a PCP-treated male mouse, (c) a saline-treated female mouse and (d) a saline-treated male mouse. Images were taken at x10 magnification. * shows location of the muscle layer.

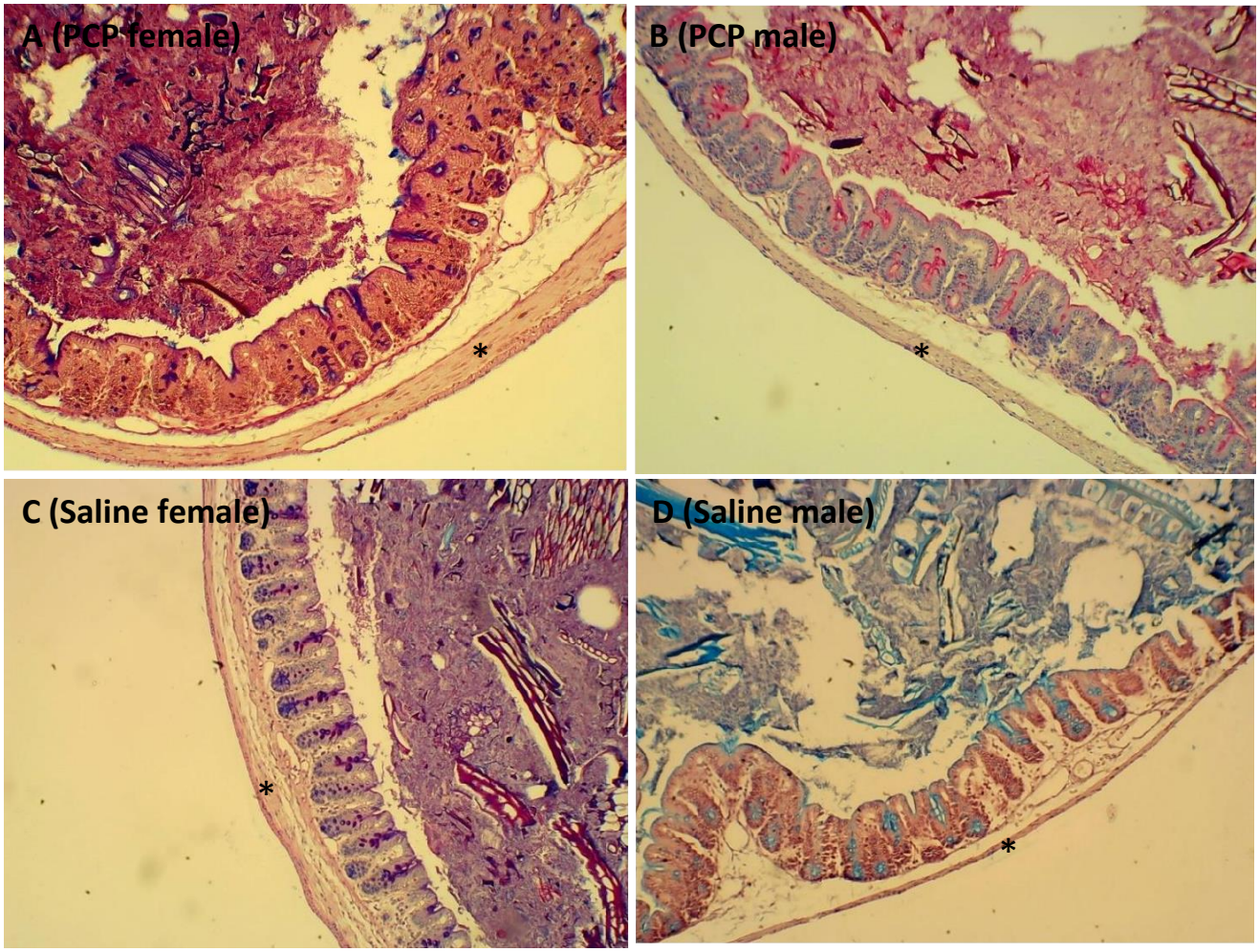


Figure 5: Representative PAS/AB stained caecum sections. Muscle layer section from (a) PCP-treated female mouse, (b) a PCP-treated male mouse, (c) a saline-treated female mouse and (d) a saline-treated male mouse. Images were taken at x10 magnification. * shows location of the muscle layer.

3.1.1.2 Subchronic PCP treatment does not significantly impact on crypt length

The lengths of crypts in the colon and caecum were viewed and imaged microscopically then measured using ImageJ software. The mean length for 20 crypts per animal was determined. Analysis showed that there was no significant difference in crypt length between subchronic PCP-treated animals and saline-treated controls in either the colon ($p = 0.693$, $F_{(1,12)} = 0.164$, Figure 6a) or in the caecum ($p = 0.989$, $F_{(1,12)} = 0.000$, Figure 6c). Furthermore, we found no significant evidence that sex significantly impacted on the length of crypts in the colon ($p = 0.791$, $F_{(1,12)} = 0.073$, Figure 6b) or in the caecum ($p = 0.455$, $F_{(1,12)} = 0.596$, Figure 6d). Finally, there was no significant influence of sex on the impact of subchronic PCP treatment in the colon ($p = 0.213$, $F_{(1,12)} = 1.733$) or in the caecum ($p = 0.117$, $F_{(1,12)} = 2.860$). Representative PAS/AB stained images are shown in Figure 7 and Figure 8.

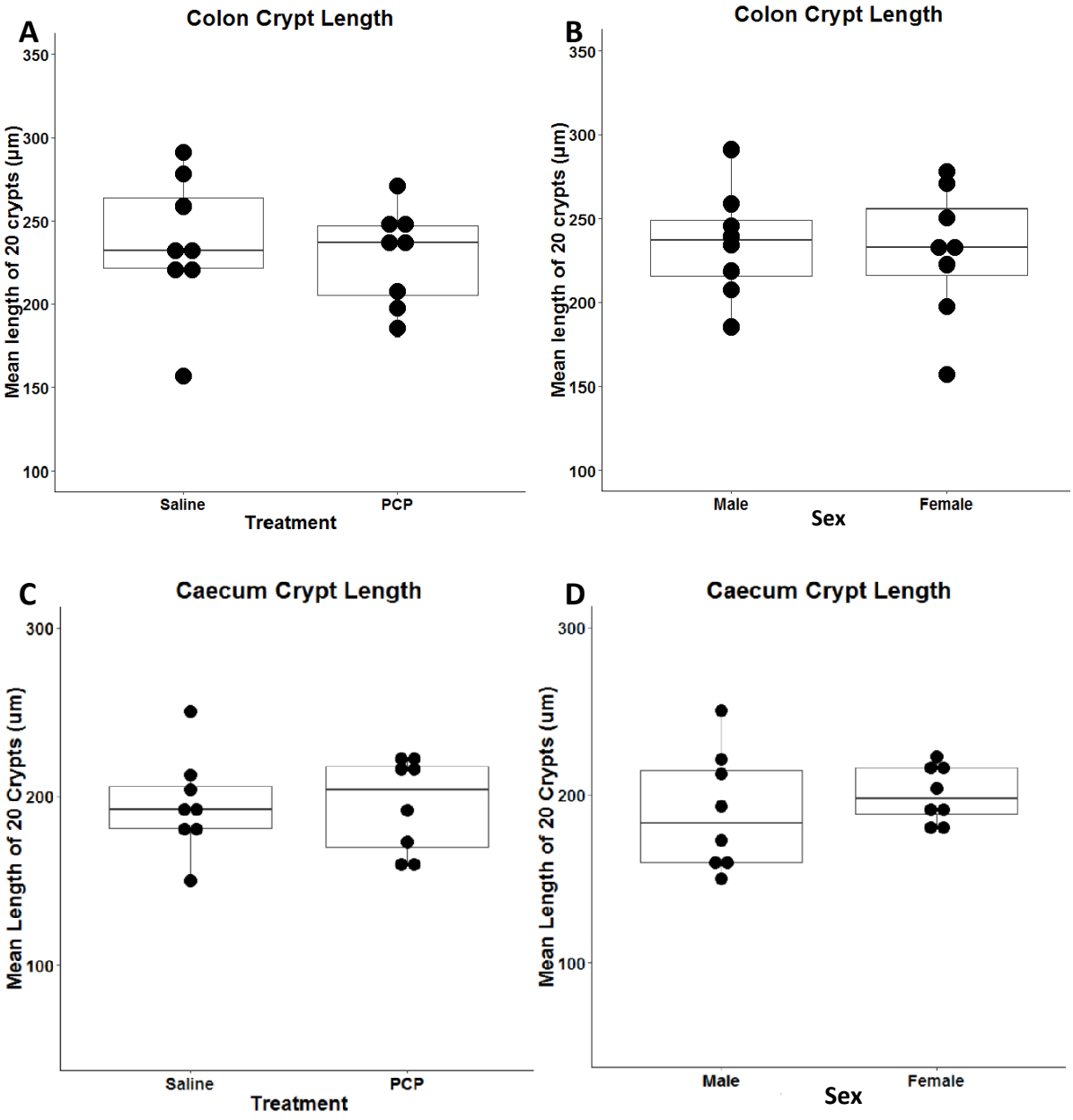


Figure 6: Subchronic PCP treatment does not impact on crypt length in the colon or caecum. (a) Colon crypt length (µm) in saline and PCP-treated mice (saline n = 8, PCP n = 8, p = 0.693, $F_{(1,12)} = 0.164$). (b) Colon crypt length in female and male mice (females n = 8, males n = 8, p = 0.791, $F_{(1,12)} = 0.073$). (c) Caecum crypt length in saline and PCP-treated mice (saline n = 8, PCP n = 8, p = 0.989, $F_{(1,12)} = 0.000$). (d) Caecum crypt length in female and male mice (females n = 8, males n = 8, p = 0.455, $F_{(1,12)} = 0.596$).

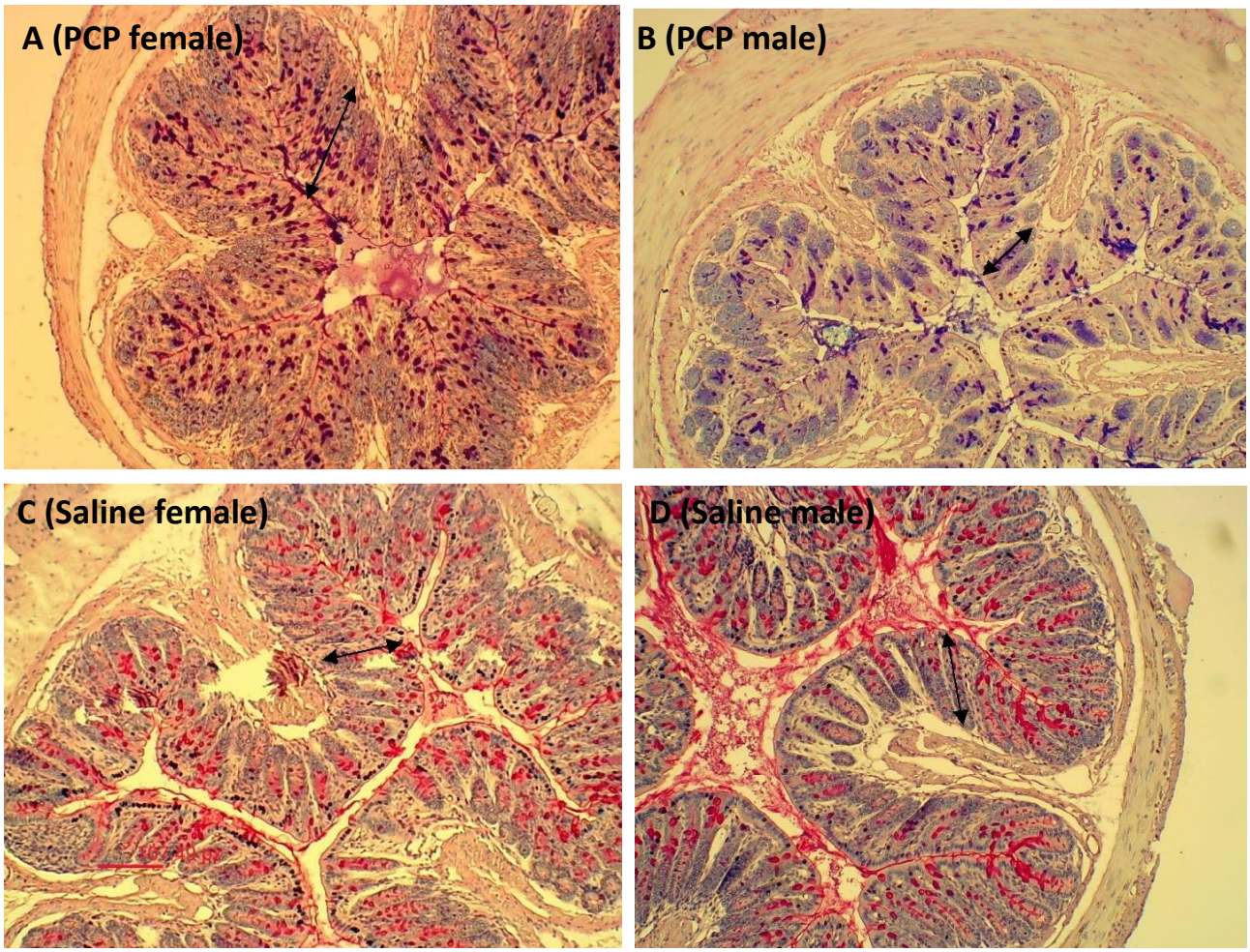


Figure 7: Representative PAS/AB stained images for crypts in the colon. Images showing (a) a PCP-treated female, (b) a PCP-treated male, (c) a saline-treated female, and (d) a saline-treated male. Images were taken at x10 magnification. **<->** represents the presence of a colon crypt.

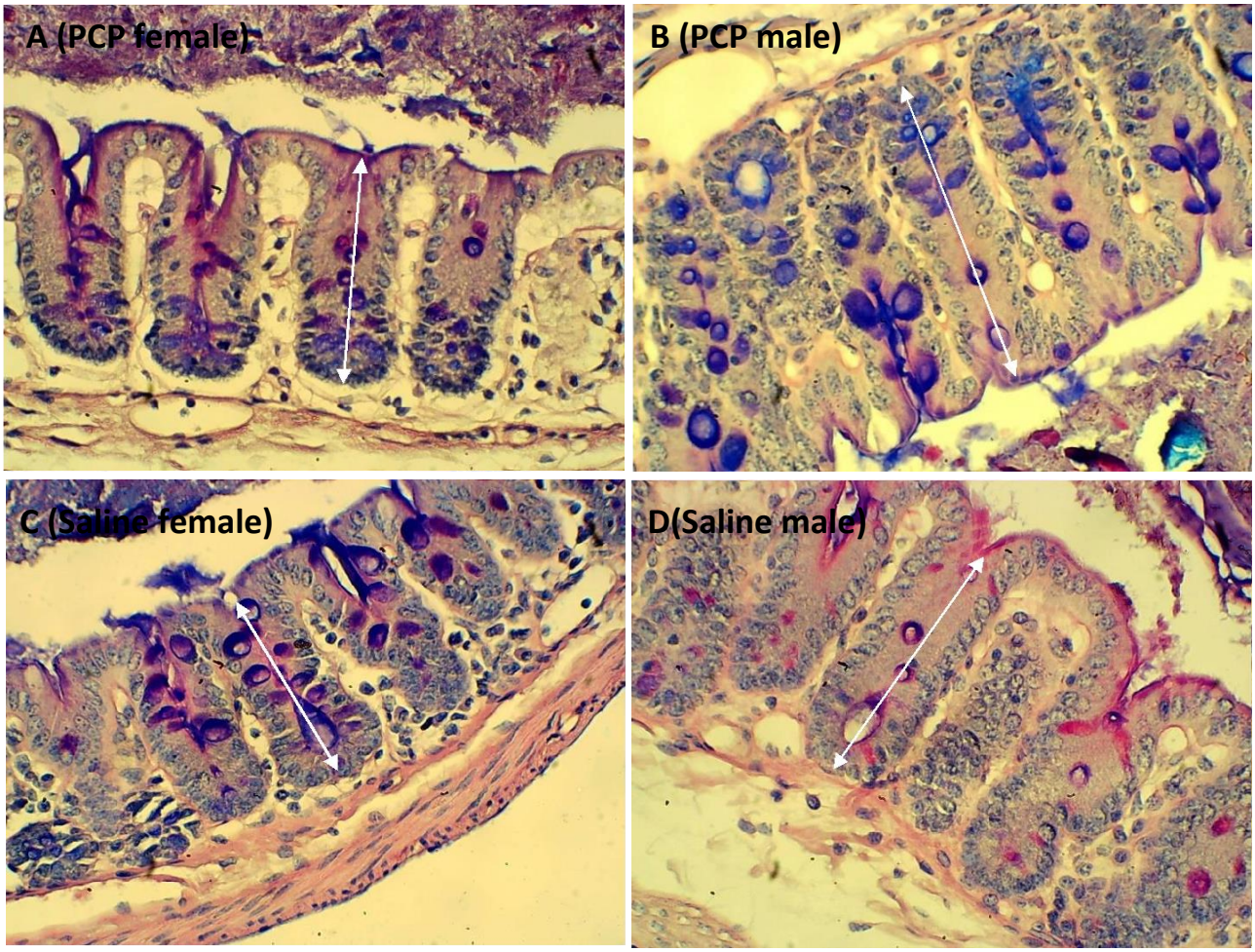


Figure 8: Representative PAS/AB stained images for crypts in the caecum. Images showing (a) a PCP-treated female, (b) a PCP-treated male, (c) a saline-treated female, and (d) a saline-treated male. These images were taken at x40 magnification. **<->** represents the presence of a colon crypt.

3.1.1.3 Subchronic PCP treatment does not impact on the size and number of goblet cells

Goblet cell size

The number of goblet cells per 20 crypts in each colon and caecum sample were counted microscopically and their mean area (μm^2) was analysed using ImageJ software. Both the top layer and bottom layers of colon and caecum crypts were considered separately due to significant size variations in goblet cells between these localisations (Shearman and Muir, 1960). Analysis shows that there was no significant difference between subchronic PCP-treated animals and saline-treated controls in the size of goblet cells in the top layer ($p = 0.326$, $F_{(1,12)} = 1.050$, Figure 9a) or bottom layer ($p = 0.132$, $F_{(1,11)} = 2.645$, Figure 9c) of the colon. In addition, we found no significant evidence that sex significantly impacted on the size of the goblet cells in the top layer ($p = 0.730$, $F_{(1,12)} = 0.125$, Figure 9b) or in the bottom layer ($p = 0.654$, $F_{(1,11)} = 0.213$, Figure 9d) of the colon. We also found that sex did not influence the impact of subchronic PCP treatment in the top layer ($p = 0.445$, $F_{(1,12)}=0.623$) or bottom layer ($p = 0.492$, $F_{(1,11)} = 0.506$) of the colon. Representative PAS/AB stained images, taken at x40 magnification, are shown in Figure 12.

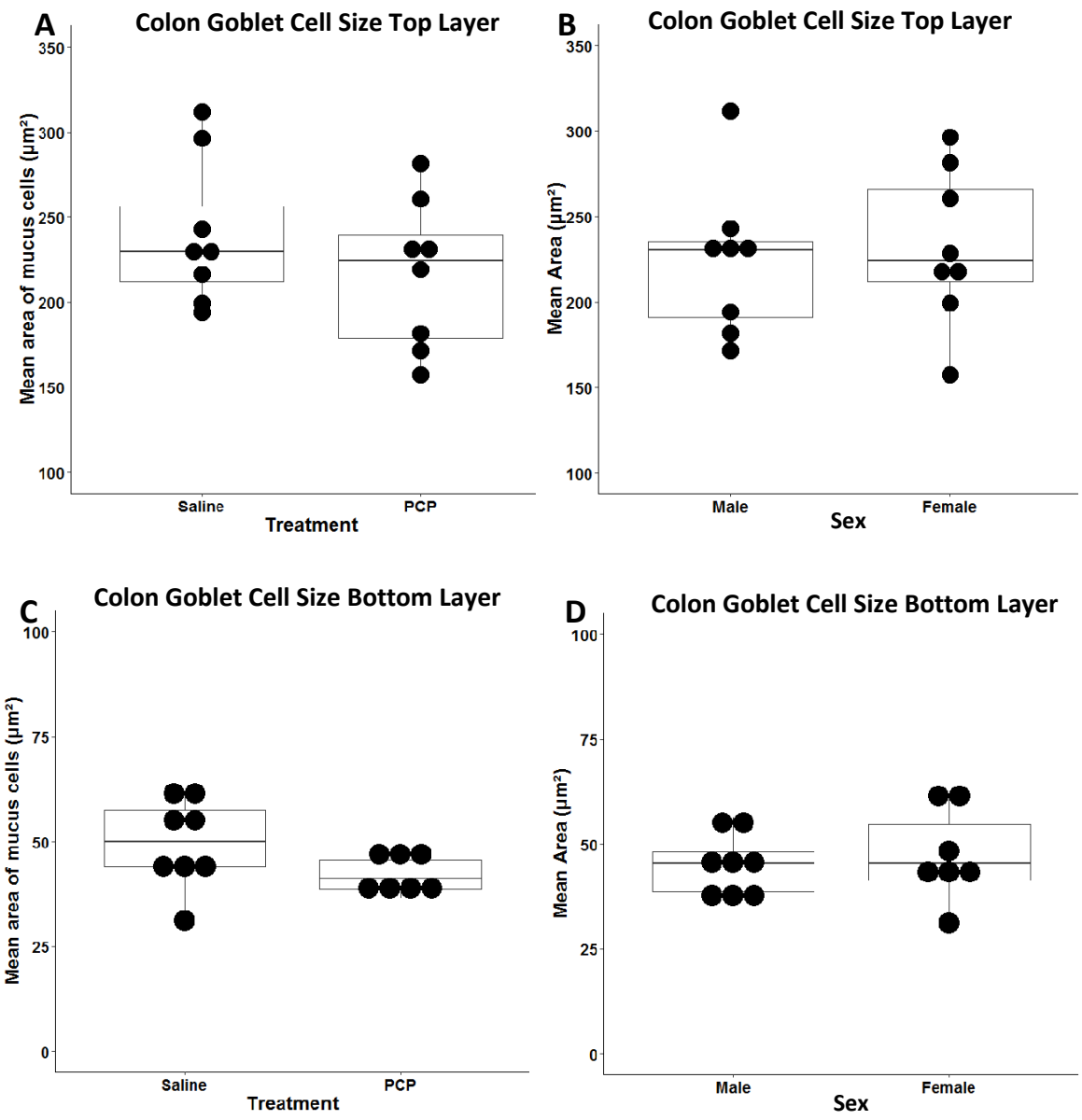


Figure 9: Subchronic PCP treatment and sex do not impact on goblet cell size in the colon. (a) colon goblet cell size (μm^2) in saline and PCP-treated mice in the top layer of colon crypts (saline n = 8, PCP n = 8, p = 0.326, $F_{(1,12)} = 1.050$). (b) goblet cell size in top layer of colon crypts in female and male mice (female n = 8, male n = 8, p = 0.730, $F_{(1,12)} = 0.125$). (c) goblet cell size in saline and PCP-treated mice in the bottom layer of colon crypts (p = 0.132, $F_{(1,11)} = 2.645$). (d) goblet cell size in bottom layer of colon crypts in male and female mice (p = 0.654, $F_{(1,11)} = 0.213$).

Analysis of goblet cell size in the caecum showed that there was no significant difference between subchronic PCP-treated animals and saline-treated controls in either the top layer ($p = 0.076$, $F_{(1,12)} = 0.101$, Figure 10a) or bottom layer ($p = 0.960$, $F_{(1,12)} = 0.003$, Figure 10c) of crypts in the caecum. We also found no significant effect of sex on the size of goblet cells in the top layer ($p = 0.071$, $F_{(1,12)} = 3.913$, Figure 10b) or bottom layer ($p = 0.452$, $F_{(1,12)} = 0.604$, Figure 10d) of crypts in the caecum. We also found that sex did not significantly influence the impact of subchronic PCP treatment in the top layer ($p = 0.679$, $F_{(1,12)} = 0.180$) or bottom layer ($p = 0.390$, $F_{(1,12)} = 0.797$) of crypts in the caecum. Representative PAS/AB stained images, taken at x40 magnification, are shown in Figure 13.

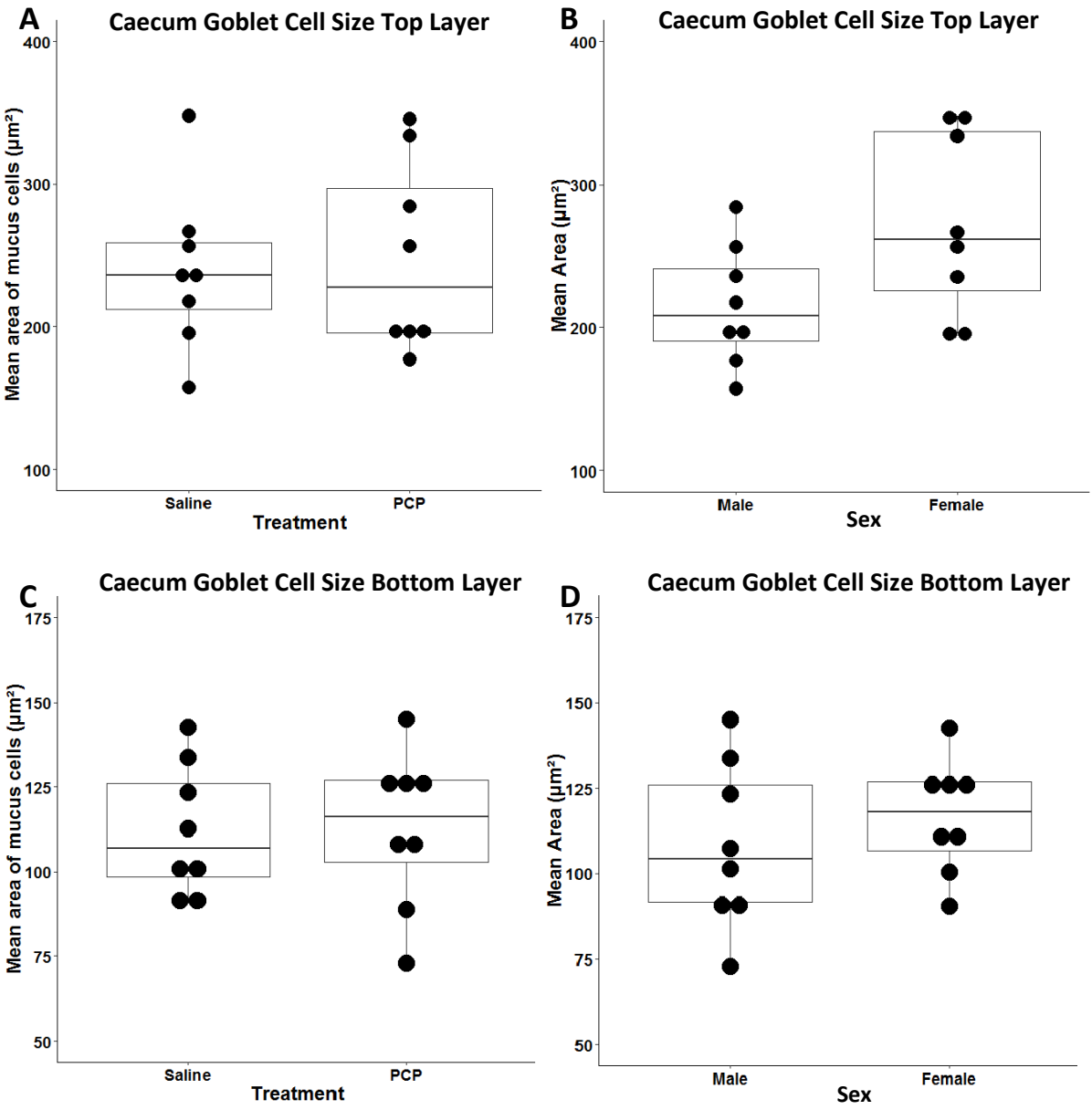


Figure 10: Subchronic PCP treatment and sex do not impact on goblet cell size in the caecum. (a) caecum goblet cell size (μm^2) in saline and PCP-treated mice in the top layer of caecum crypts (saline n = 8, PCP n = 8, p = 0.076, $F_{(1,12)} = 0.101$). (b) goblet cell size in top layer of caecum crypts in females and males (female n = 8, male n = 8, p = 0.071, $F_{(1,12)} = 3.913$). (c) goblet cell size in saline and PCP-treated mice in bottom layer of caecum crypts (p = 0.960, $F_{(1,12)} = 0.003$). (d) goblet cell size in bottom layer of caecum crypts in males and females (p = 0.452, $F_{(1,12)} = 0.604$).

Goblet cell number

Further analysis of goblet cells determined the mean goblet cell number per 20 crypts in the colon and caecum. We found no significant differences between subchronic PCP-treated animals and saline treated controls in the colon ($p = 0.124$, $F_{(1,12)} = 2.729$, Figure 11a) or in the caecum ($p = 0.316$, $F_{(1,12)} = 1.096$, Figure 11c). There was no effect of sex on the mean goblet cell number in the colon ($p = 0.074$, $F_{(1,12)} = 3.812$, Figure 11b) or in the caecum ($p = 0.216$, $F_{(1,12)} = 1.708$, Figure 11d). We also found no significant evidence that sex influenced on the impact of subchronic PCP treatment on the mean goblet cell number in the colon ($p = 0.609$, $F_{(1,12)} = 0.276$) or in the caecum ($p = 0.118$, $F_{(1,12)} = 2.837$).

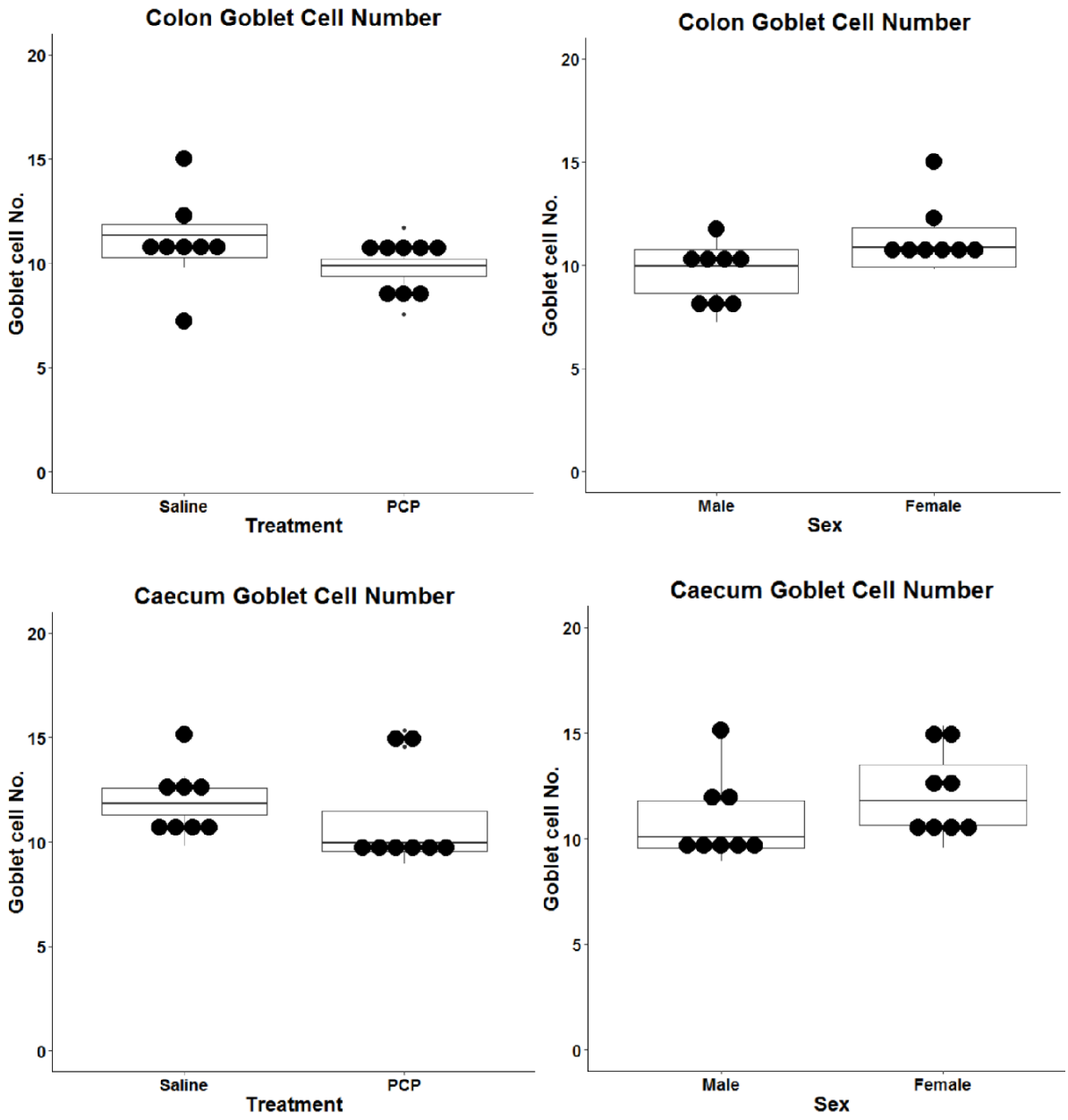


Figure 11: Subchronic PCP treatment and sex do not impact on goblet cell number in the colon or caecum. Data shows the mean goblet cell number per 20 crypts analysed. (a) colon goblet cell number in saline and PCP-treated mice (saline n = 8, PCP n = 8, p = 0.124, $F_{(1,12)} = 2.729$). (b) goblet cell number in the colon of female and male mice (female n = 8, male n = 8, p = 0.075, $F_{(1,12)} = 3.812$). (c) caecum goblet cell number in saline and PCP-treated mice (p = 0.316, $F_{(1,12)} = 1.096$). (d) goblet cell number in the caecum of female and male mice (p = 0.216, $F_{(1,12)} = 1.708$).

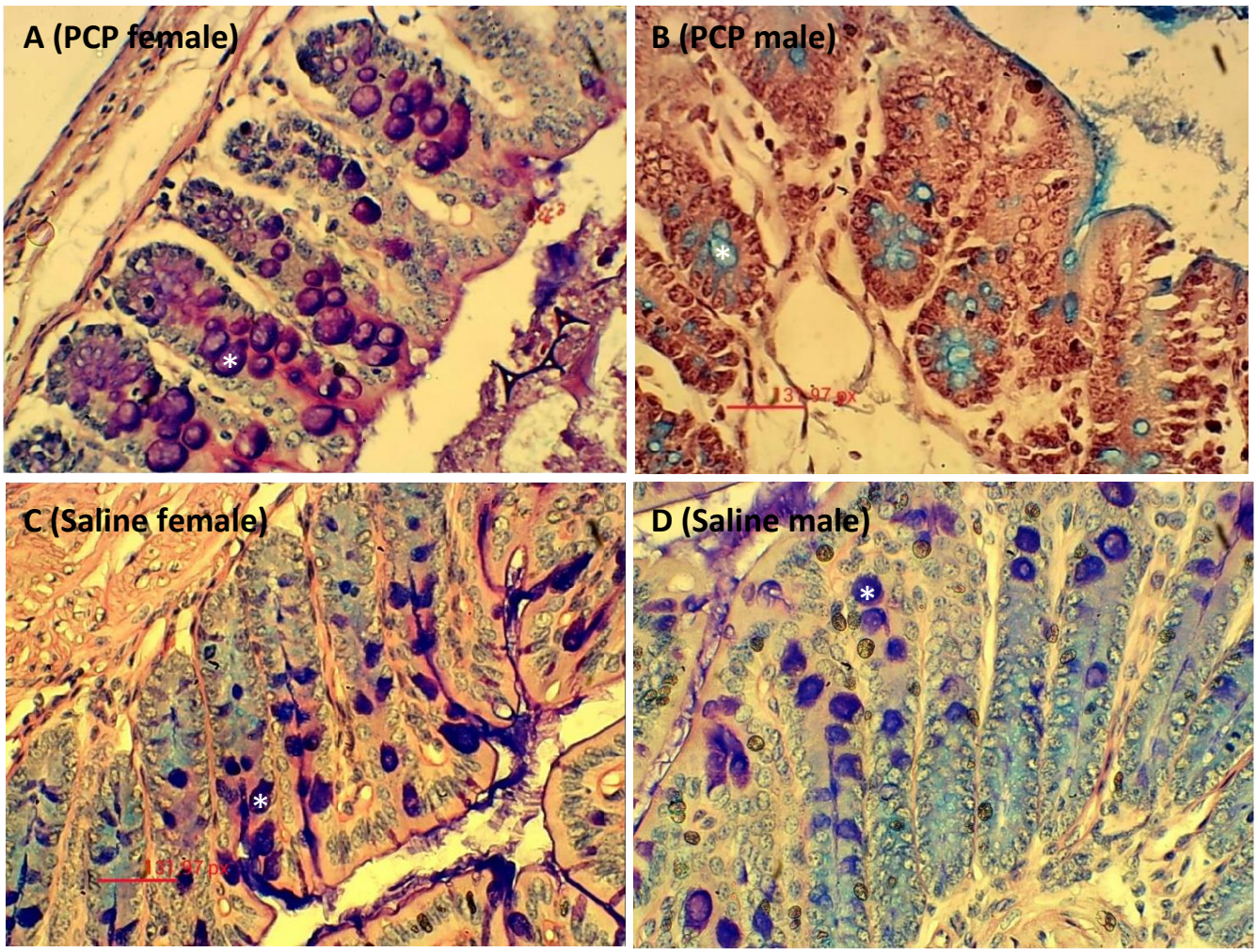


Figure 12: Representative PAS/AB stained images showing goblet cells in the colon. Images showing goblet cells in (a) a PCP-treated female, (b) a PCP-treated male, (c) a saline-treated female, and (d) a saline-treated male. Images were taken at x40 magnification. * indicates the presence of a goblet cell.

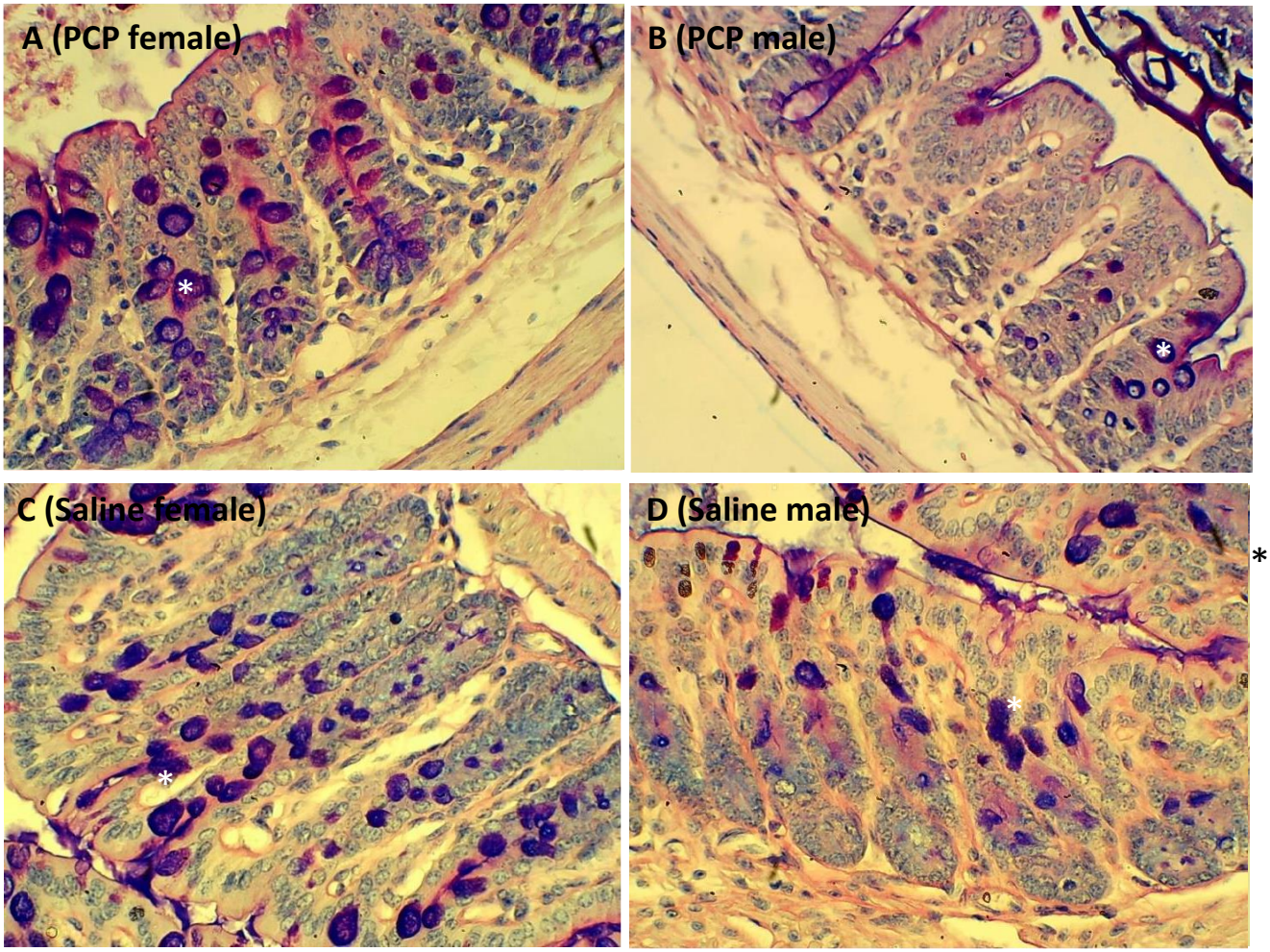


Figure 13: Representative PAS/AB stained images showing goblet cells in the caecum. Images showing goblet cells in (a) a PCP-treated female, (b) a PCP-treated male, (c) a saline-treated female, and (d) a saline-treated male. Images were taken at x40 magnification. * indicates the presence of a goblet cell.

3.1.1.4 Subchronic PCP treatment does not significantly impact on EEC numbers

The number of EECs in the colon and caecum were determined using fluorescent microscope of sections stained using an antibody raised against CgA (anti-CgA, Ab15160) with a secondary antibody conjugated to 488 donkey anti-rabbit IgG, and DAPI stain. Colon and caecum sections were analysed to count the average total number of EECs present per 20 crypts in the colon and caecum. There was no significant difference between subchronic PCP-treated animals and saline-treated controls in the average number of EECs in the colon ($p = 0.902$, $F(1,11) = 0.016$, Figure 14a) or in the caecum ($p = 0.173$, $F(1,12) = 2.101$, Figure 14c). There was also no effect of sex on the number of EECs present in the colon ($p = 0.660$, $F(1,11) = 0.204$, Figure 14b) or in the caecum ($p = 0.385$, $F(1,12) = 0.812$, Figure 14d). We also found no significant evidence that sex influences the impact of subchronic PCP-treatment on EEC cell number in the colon ($p = 0.751$, $F(1,11) = 0.106$) or in the caecum ($p = 0.195$, $F(1,12) = 1.880$). Representative anti-CgA/DAPI stained images, taken at x40 magnification, are shown in Figure 15 and Figure 16.

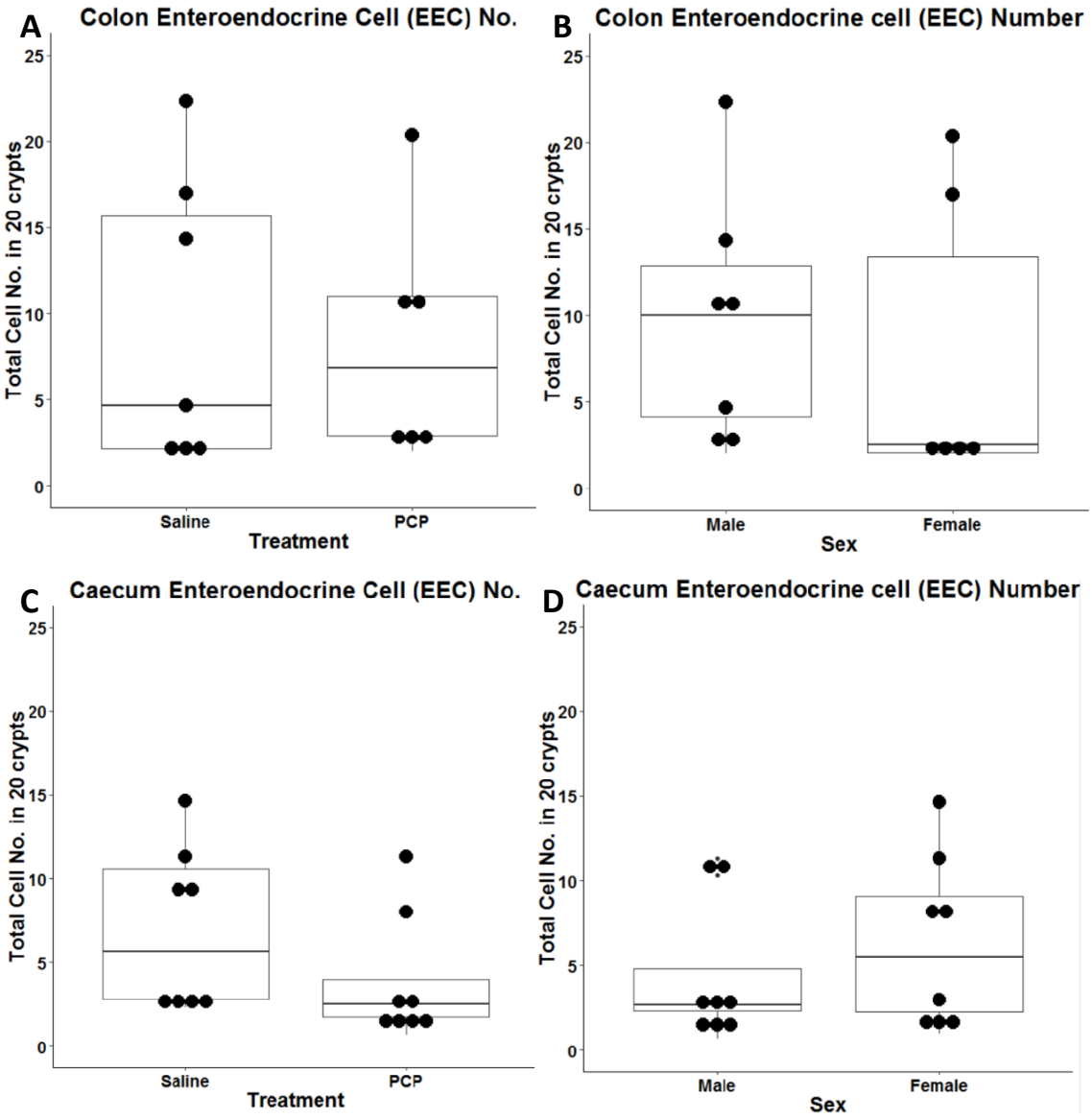


Figure 14: Subchronic PCP treatment and sex do not impact on EEC number in the colon or caecum. (a) Colon EEC number in saline and PCP-treated mice (saline n = 7, PCP n = 6, p = 0.902, $F_{(1,11)} = 0.016$). (b) Colon EEC number in female and male mice (females n = 7, males n = 6, p = 0.660, $F_{(1,11)} = 0.204$). (c) Caecum EEC number in saline and PCP-treated mice (saline n = 8, PCP n = 8, p = 0.173, $F_{(1,12)} = 2.101$). (d) Caecum EEC number in female and male mice (females n = 8, males n = 8, p = 0.385, $F_{(1,12)} = 0.812$).

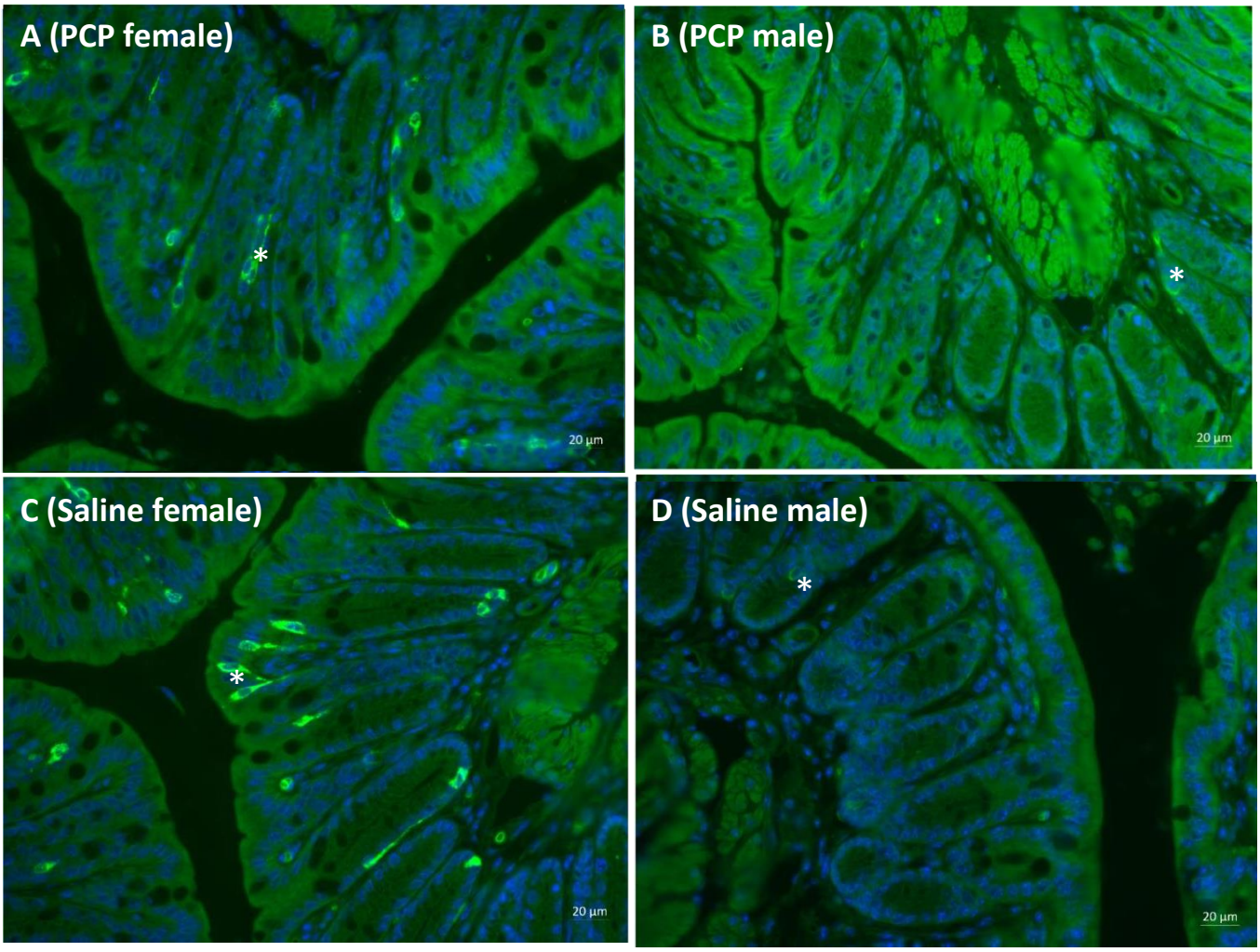


Figure 15: Representative anti-CgA/DAPI stained images for EEC's in the colon. Images showing (a) a PCP-treated female, (b) a PCP-treated male, (c) a saline-treated female, and (d) a saline-treated male. Ab15160 conjugated to 488 donkey anti-rabbit IgG binds to CgA in EECs (bright green).; DAPI is a fluorescent stain that binds strongly to adenine–thymine rich regions in DNA, present in the nucleus (blue). Images were taken at x40 magnification. * indicates the presence of an EEC (bright green).

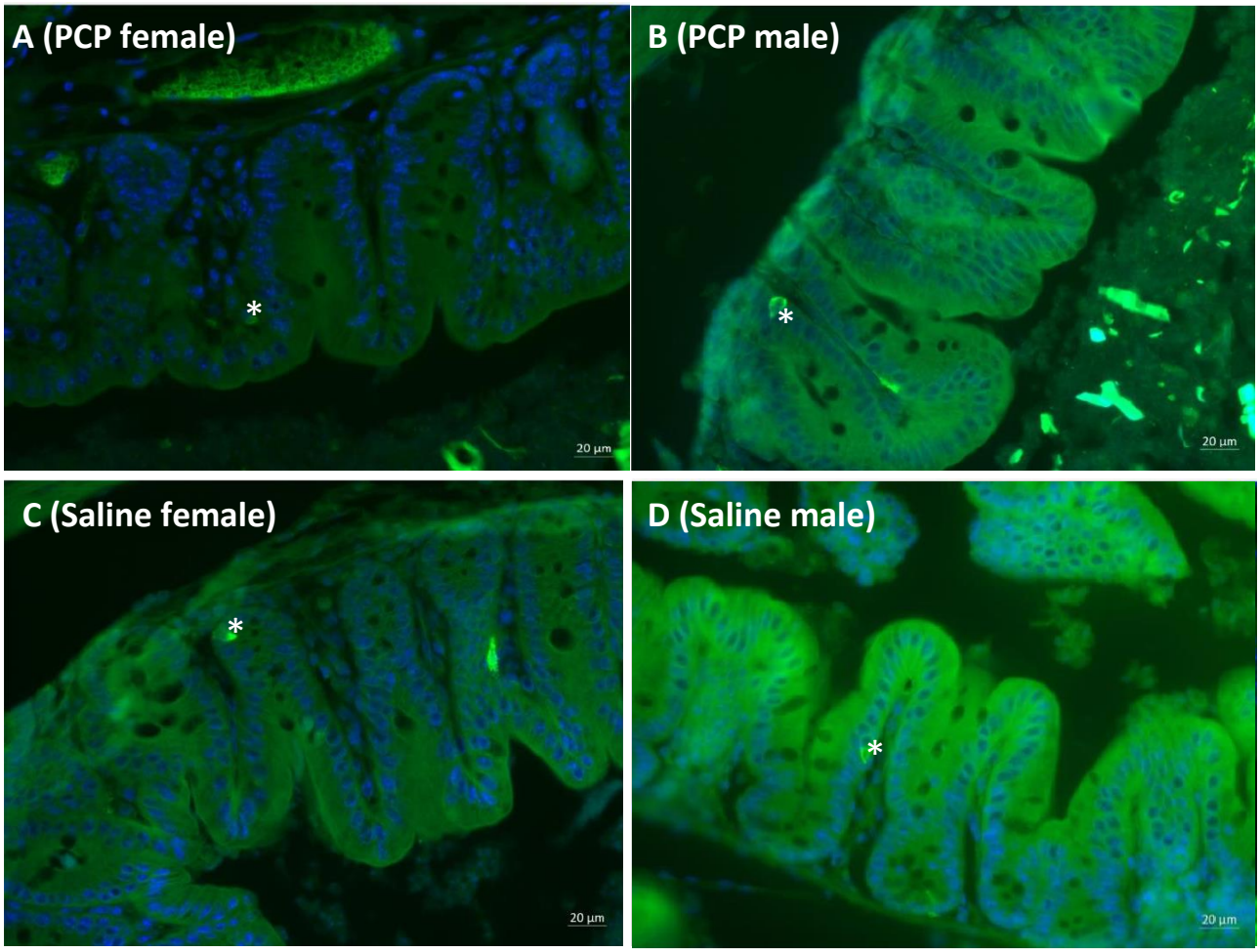


Figure 16: Representative anti-CgA/DAPI stained images for EECs in the caecum. Images showing (a) a PCP-treated female, (b) a PCP-treated male, (c) a saline-treated female, and (d) a saline-treated male. Images were taken at x40 magnification. * indicates the presence of an EEC (bright green).

3.1.1.5 Summary: Impact of subchronic PCP treatment on gut architecture

Subchronic PCP treatment increases the depth of the muscle layer in the colon but not in the caecum. There was no evidence that PCP treatment has a significant effect on the number, or the size, of the mucus-secreting goblet cells, crypt length, or the number of EECs in the colon or the caecum. We found no evidence that the impact of subchronic PCP on gut architecture was significantly influenced by sex.

With regards to sex itself, we found that female mice had a greater muscle layer depth than males in the caecum, suggesting that there are sex differences in relation to the amount of muscle in the region. No other significant sex-related effects on gut architecture were significant.

3.1.2 Impact of *Neurexin1α* heterozygosity

Neurexin1α heterozygous (*Nrxn1α*$^{+/-}$) and wild-type mice were treated with either liraglutide or saline (see Methods section 2.2.1) and their gut architecture was examined microscopically using the same histological stains previously outlined. As *Nrxn1α* gene heterozygosity increases the risk of developing both schizophrenia and autism (Rujescu et al, 2009), it can be used as a representative model to examine the gut architecture in a model of schizophrenia. As Liraglutide treatment did not significantly interact with genotype or sex, animals treated with Liraglutide were still pooled when investigating the impact of *Nrxn1α* heterozygosity and are still represented as points on subsequent figures, which contribute to the n-values observed. There is some variation with respect to the n-values due to outliers and these were therefore removed from the analysis.

3.1.2.1 *Neurexin1α* heterozygosity decreases muscle layer depth in the colon and caecum

Nrxn1α heterozygosity significantly decreases the depth of the muscle layer in both the colon ($p = 0.003$, $F_{(1,29)} = 10.222$, Figure 17a) and caecum ($p = 0.012$, $F_{(1,25)} = 7.408$, Figure 17c). In this study, sex also had a significant effect on the depth of the muscle layer in the caecum; with females have a significantly thicker muscle layer than males ($p = 0.026$, $F_{(1,25)} = 5.575$, Figure 17d). This sex effect reproduced that found in the previous study characterising the impact of subchronic PCP treatment. No sex effects on the muscle layer depth were seen in the colon ($p = 0.738$, $F_{(1,29)} = 0.114$, Figure 17b). The impact of *Nrxn1α* heterozygosity on the muscle layer depth was not significantly influenced by sex in the colon ($p = 0.669$, $F_{(1,29)} = 0.187$) or in the caecum ($p = 0.696$, $F_{(1,25)} = 0.157$), as we found no significant sex x genotype interactions. Representative PAS/AB stained images, taken at x10 magnification, are shown in Figure 18 and Figure 19.

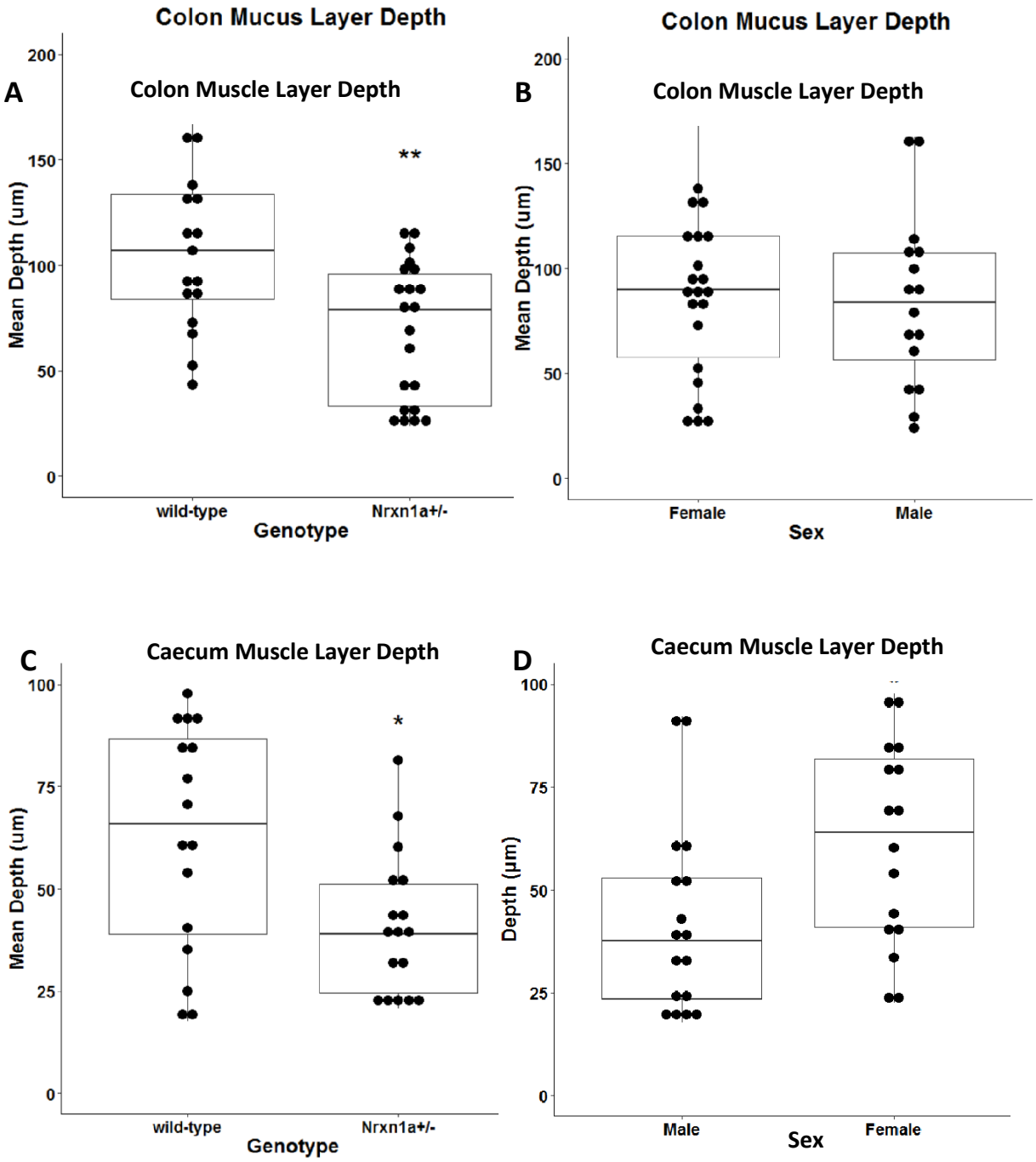


Figure 17: *Nrxn1α*$^{+/-}$ mice have a thinner muscle layer in both the colon and caecum. (a) colon muscle layer depth (µm) in wild-type and *Nrxn1α*$^{+/-}$ mice (wild-type n = 17, *Nrxn1α*$^{+/-}$ n = 21, p = 0.003, $F_{(1,29)}$ = 10.222). (b) colon muscle layer depth in female and male mice (female n = 22, male n = 16, p = 0.738, $F_{(1,29)}$ = 0.114). (c) caecum muscle layer depth in wild-type and *Nrxn1α*$^{+/-}$ mice (wild-type n = 16, *Nrxn1α*$^{+/-}$ n = 17, p = 0.012, $F_{(1,25)}$ = 7.408). (d) caecum muscle layer depth in female and male mice (wild-type n = 17, *Nrxn1α*$^{+/-}$ n = 16, p = 0.0263, $F_{(1,25)}$ = 5.575). n-values include samples from both treatment groups as there was no significant treatment x genotype interactions. *P<0.01 and **P<0.05 compared to relevant control (ANOVA).

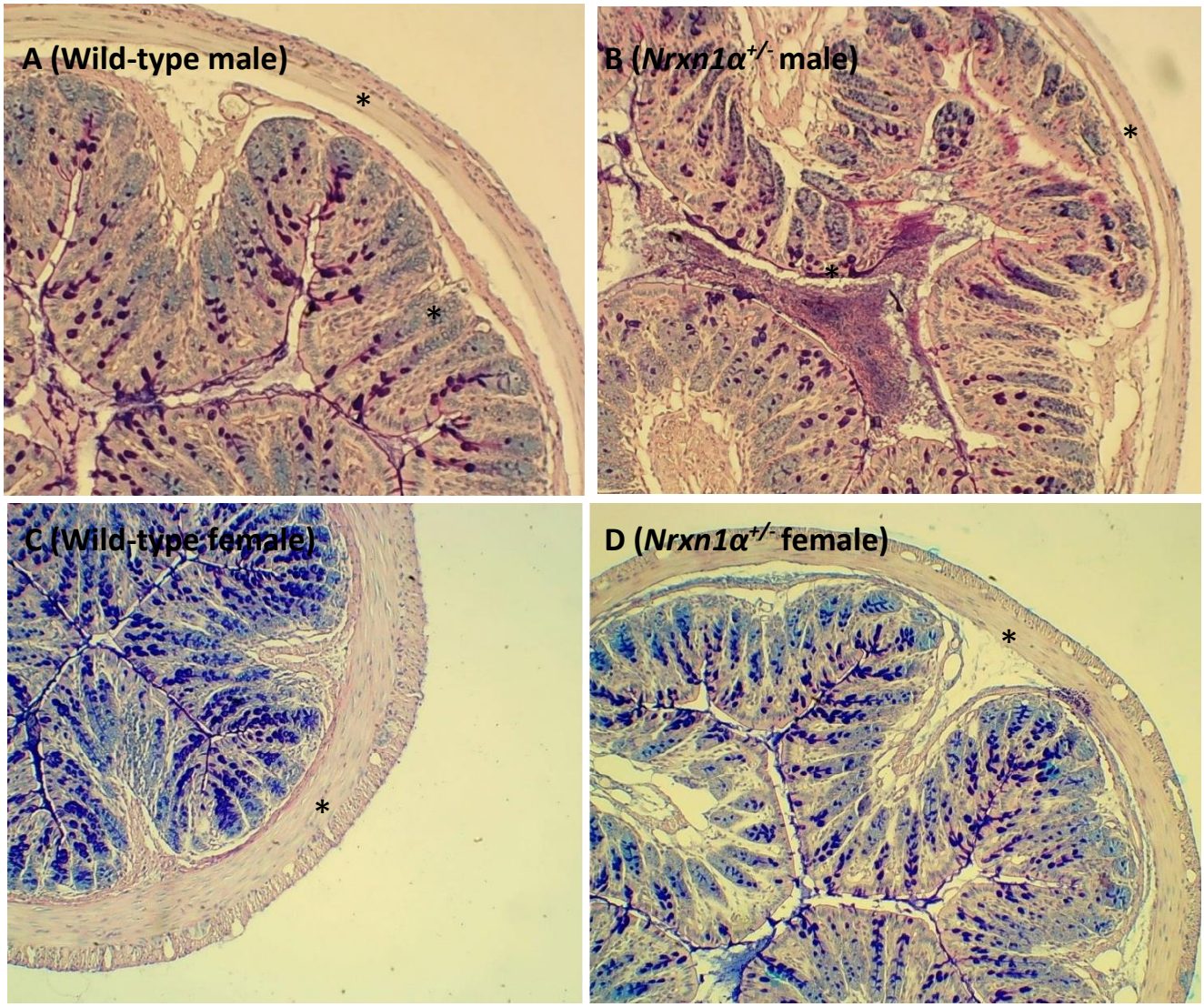


Figure 18: Representative PAS/AB stained images for muscle layer depth in the colon of *Nrxn1α*$^{+/-}$ mice.

Images showing (a) a wild-type male, (b) a *Nrxn1α*$^{+/-}$ male, (c) a wild-type female, and (d) a *Nrxn1α*$^{+/-}$ female. These mice were all treated with saline. Images were taken at x10 magnification. *indicates the location of the muscle layer.

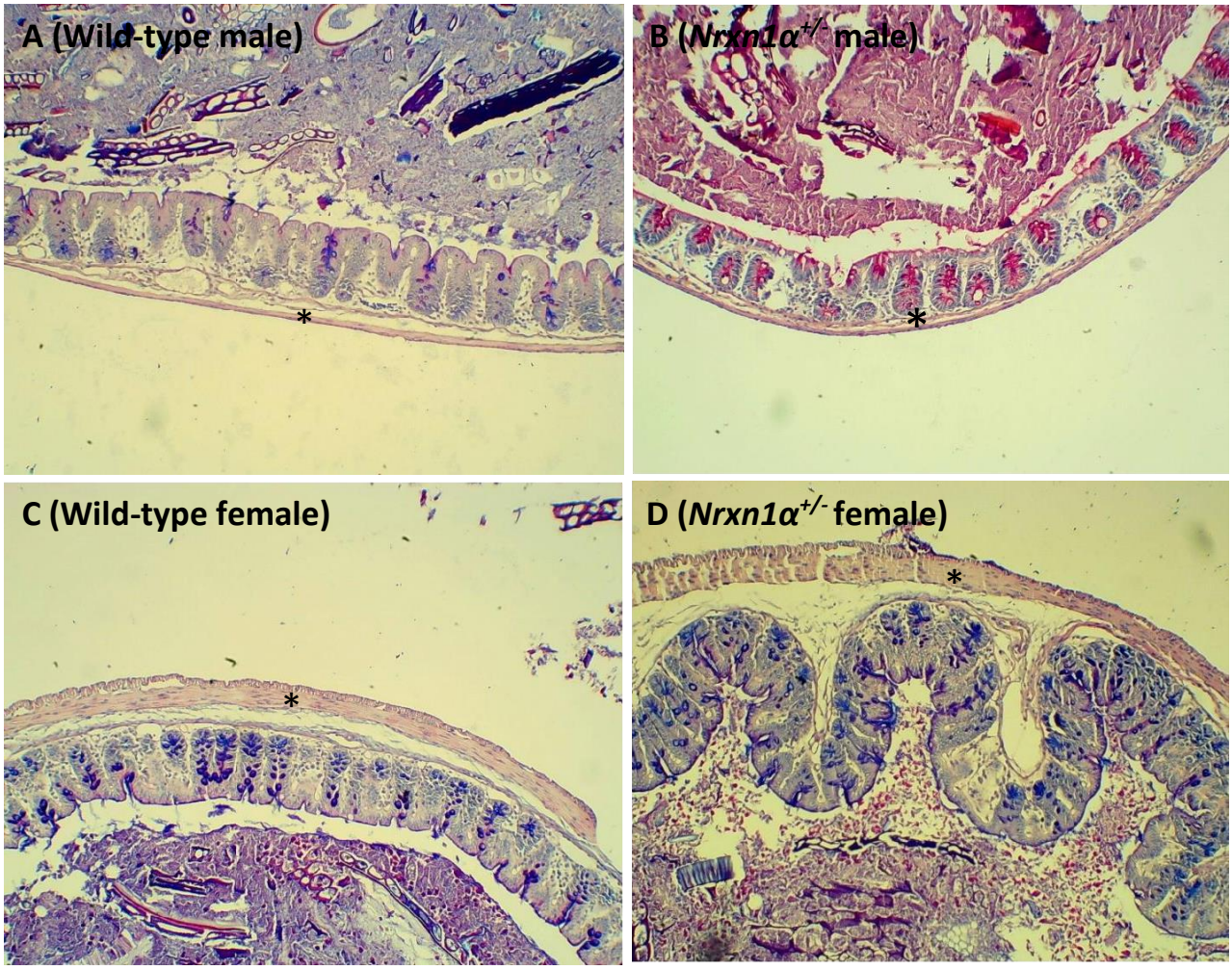


Figure 19: Representative PAS/AB stained images for muscle layer depth in the caecum. Images showing (a) a wild-type male, (b) a *Nrxn1α*$^{+/-}$ male, (c) a wild-type female, and (d) a *Nrxn1α*$^{+/-}$ female. These mice were all treated with saline. Images were taken at x10 magnification. *indicates the location of the muscle layer.

3.1.2.2 Colon crypt length is significantly shorter in *Neurexin1α*$^{+/-}$ female, but not male, mice

Neither genotype ($p = 0.077$, $F_{(1,28)} = 3.375$) nor sex ($p = 0.634$, $F_{(1,28)} = 0.231$) have an independent significant effect on the length of colon crypts, however there is a significant sex x genotype interaction ($p = 0.022$, $F_{(1,28)} = 5.886$). Female mice heterozygous for *Nrxn1α* have significantly shorter colon crypts when compared to wild-type females ($p = 0.042$, Tukey's HSD, Figure 20a). However, this effect was not seen in males ($p = 0.803$, Tukey's HSD, Figure 20b). In the caecum, crypt length was not significantly influenced by sex ($p = 0.495$, $F_{(1,10)} = 0.479$) or genotype ($p = 0.919$, $F_{(1,25)} = 0.011$) and we found no evidence for a significant sex x genotype interaction ($p = 0.930$, $F_{(1,25)} = 0.008$). These findings suggest that crypt length in the colon is only significantly affected in female mice heterozygous for *Nrxn1α*. Representative PAS/AB stained colon crypt images, taken at x10 magnification, are shown in Figure 21.

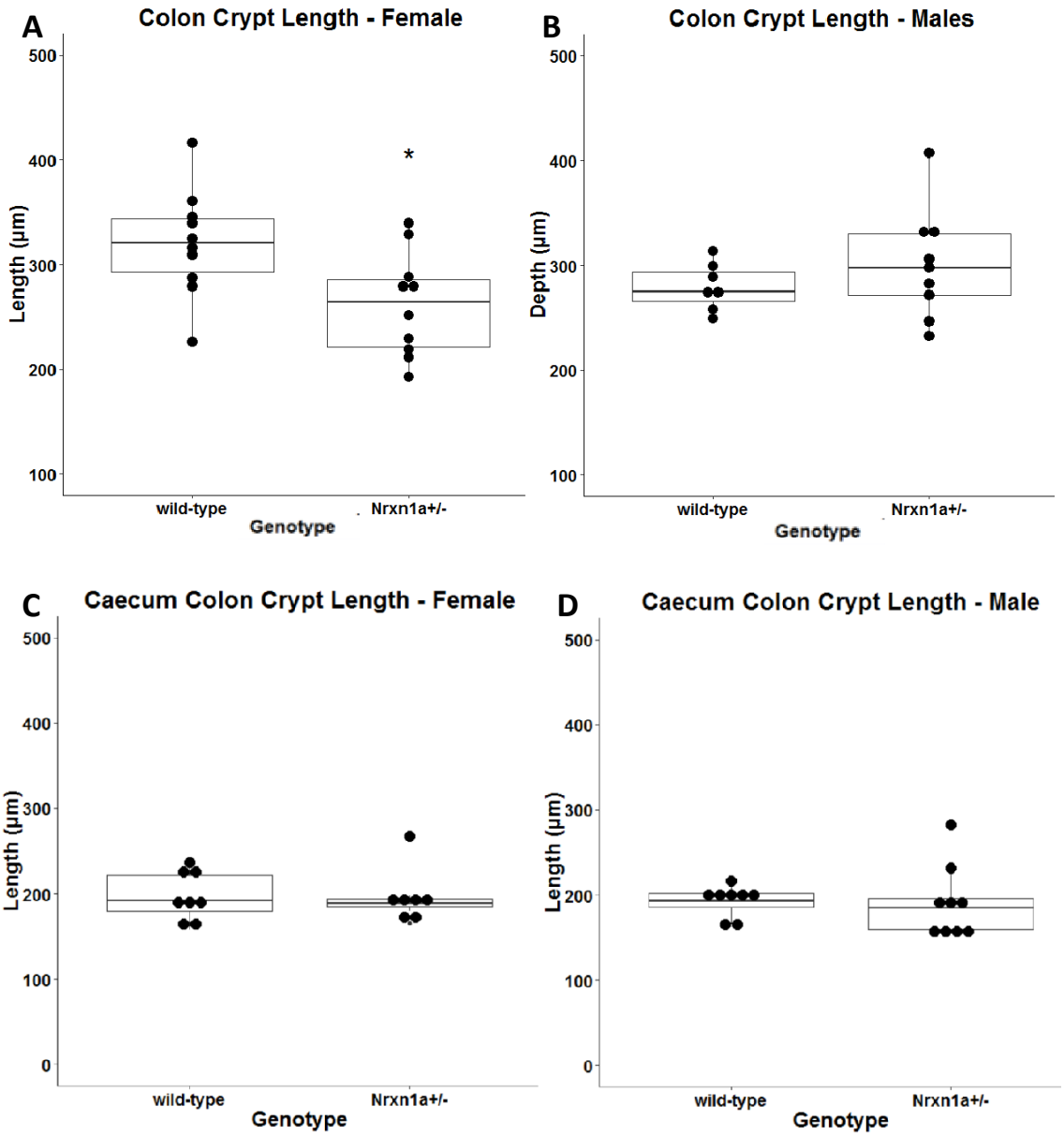


Figure 20: *Nrxn1α*$^{+/-}$ heterozygosity decreases colon crypt length in female but not in male mice. (a) Colon crypt length (µm) in wild-type and *Nrxn1α*$^{+/-}$ females (wild-type females n = 11, *Nrxn1α*$^{+/-}$ females n = 10). (b) Colon crypt length in wild-type and *Nrxn1α*$^{+/-}$ males (wild-type males n = 7, *Nrxn1α*$^{+/-}$ males n = 9). (c) Caecum crypt length (µm) in wild-type and *Nrxn1α*$^{+/-}$ females (wild-type females n = 8, *Nrxn1α*$^{+/-}$ males n = 7). (b) Caecum crypt length in wild-type and *Nrxn1α*$^{+/-}$ males (wild-type males n = 8, *Nrxn1α*$^{+/-}$ males n = 9). n-values include samples from both treatment groups as there was no significant treatment x genotype interactions. *P<0.05 compared to wild-type control (Tukey's HSD).

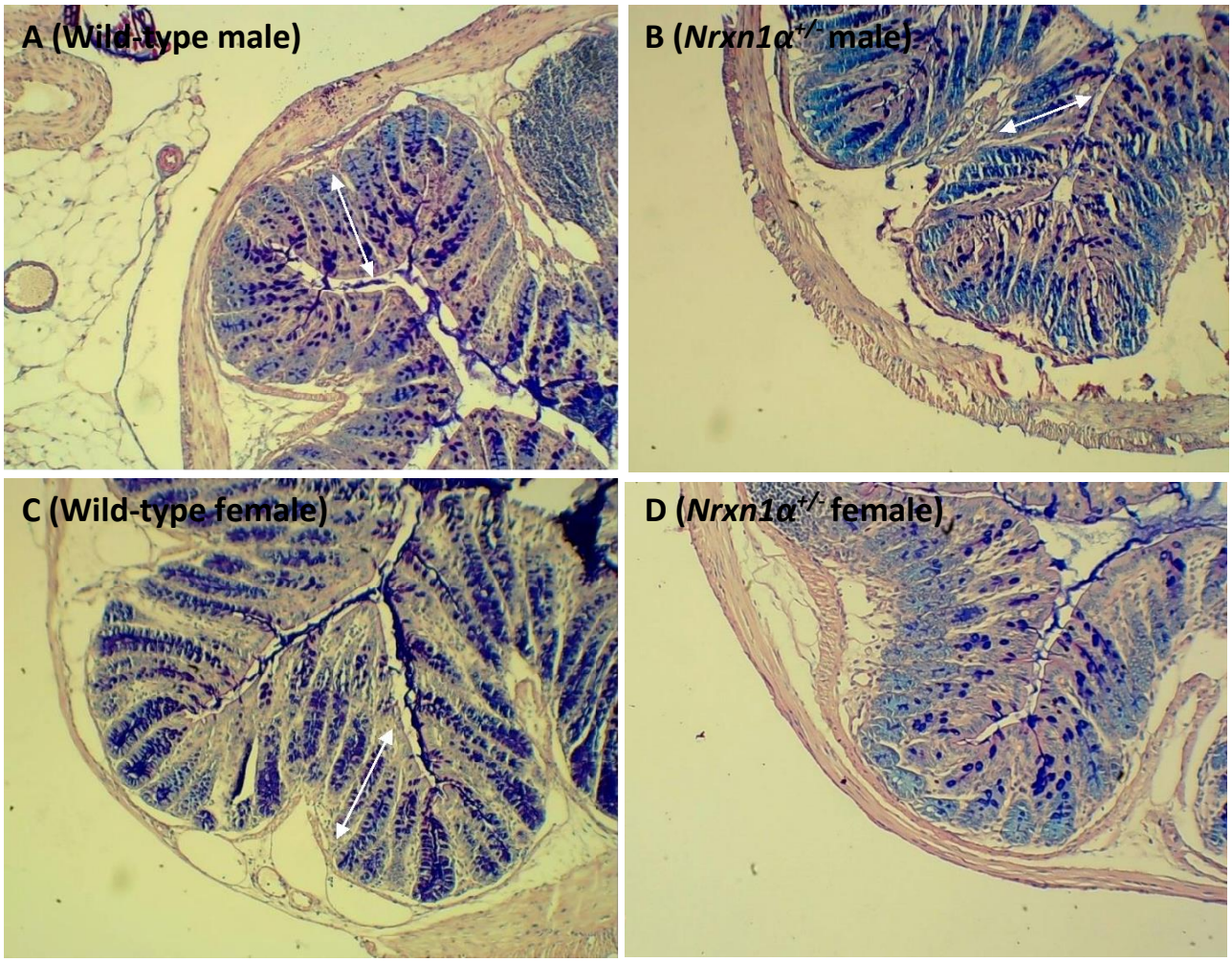


Figure 21: Representative PAS/AB stained images for crypt length in the colon. Images showing (a) a wild-type male, (b) a *Nrxn1α*$^{+/-}$ male, (c) a wild-type female, and (d) a *Nrxn1α*$^{+/-}$ female. These mice were all treated with saline. Images were taken at x10 magnification. <-> indicates the presence of a crypt.

3.1.2.3 *Neurexin1α* heterozygosity decreases goblet cell number in the colon of female, but not male, mice

We found evidence for a significant interaction between sex and genotype on the number of goblet cells counted in the colon ($p = 0.006$, $F_{(1,28)} = 8.610$). Post-hoc analysis confirmed a significant decrease in the goblet cell count in the colon of females heterozygous for *Nrxn1α*$^{+/-}$ in comparison to wild-type females ($p = 0.029$, Tukey's HSD, Figure 22a) while there was no evidence for a genotype difference in male mice ($p = 0.466$, Tukey's HSD, Figure 22b). These data parallel previous findings with regards to the decrease in crypt length seen in *Nrxn1α*$^{+/-}$ female mice colon. By contrast, no significant effects on goblet cell count were seen in the caecum with respect to sex ($p = 0.643$, $F_{(1,29)} = 0.220$) or genotype ($p = 0.483$, $F_{(1,29)} = 0.504$). We found no evidence for a sex x genotype interaction in the caecum ($p = 0.950$, $F_{(1,29)} = 0.004$, Figure 22c, Figure 22d). Representative PAS/AB stained images, taken at x40 magnification, are shown in Figure 23.

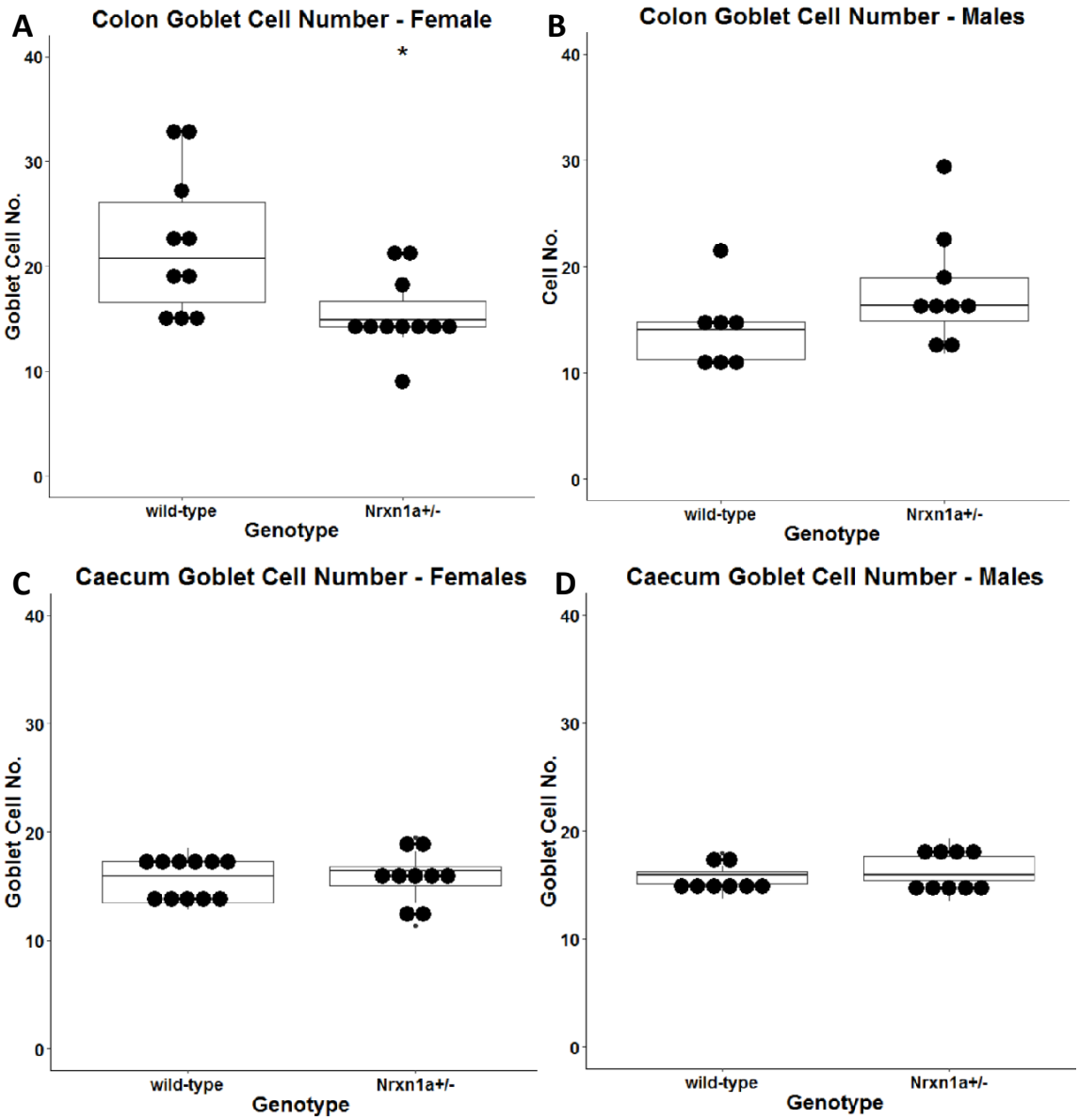


Figure 22: *Neurexin1α* heterozygosity decreases colon goblet cell number in female, but not in male, mice.

We determined the mean goblet cell number per 20 crypts analysed. (a) Colon goblet cell number in wild-type and *Nrxn1α*$^{+/-}$ females (wild-type females n = 10, *Nrxn1α*$^{+/-}$ females n = 11). (b) Colon goblet cell number in wild-type and *Nrxn1α*$^{+/-}$ males (wild-type males n = 7, *Nrxn1α*$^{+/-}$ males n = 9). (c) Caecum goblet cell number in wild-type and *Nrxn1α*$^{+/-}$ females (wild-type females n = 11, *Nrxn1α*$^{+/-}$ females n = 9). (d) Caecum goblet cell number in wild-type and *Nrxn1α*$^{+/-}$ males (wild-type males n = 8, *Nrxn1α*$^{+/-}$ males n = 9). n-values include samples from both treatment groups as there was no significant treatment x genotype interactions. *P<0.05 compared to relevant control (Tukey's HSD).

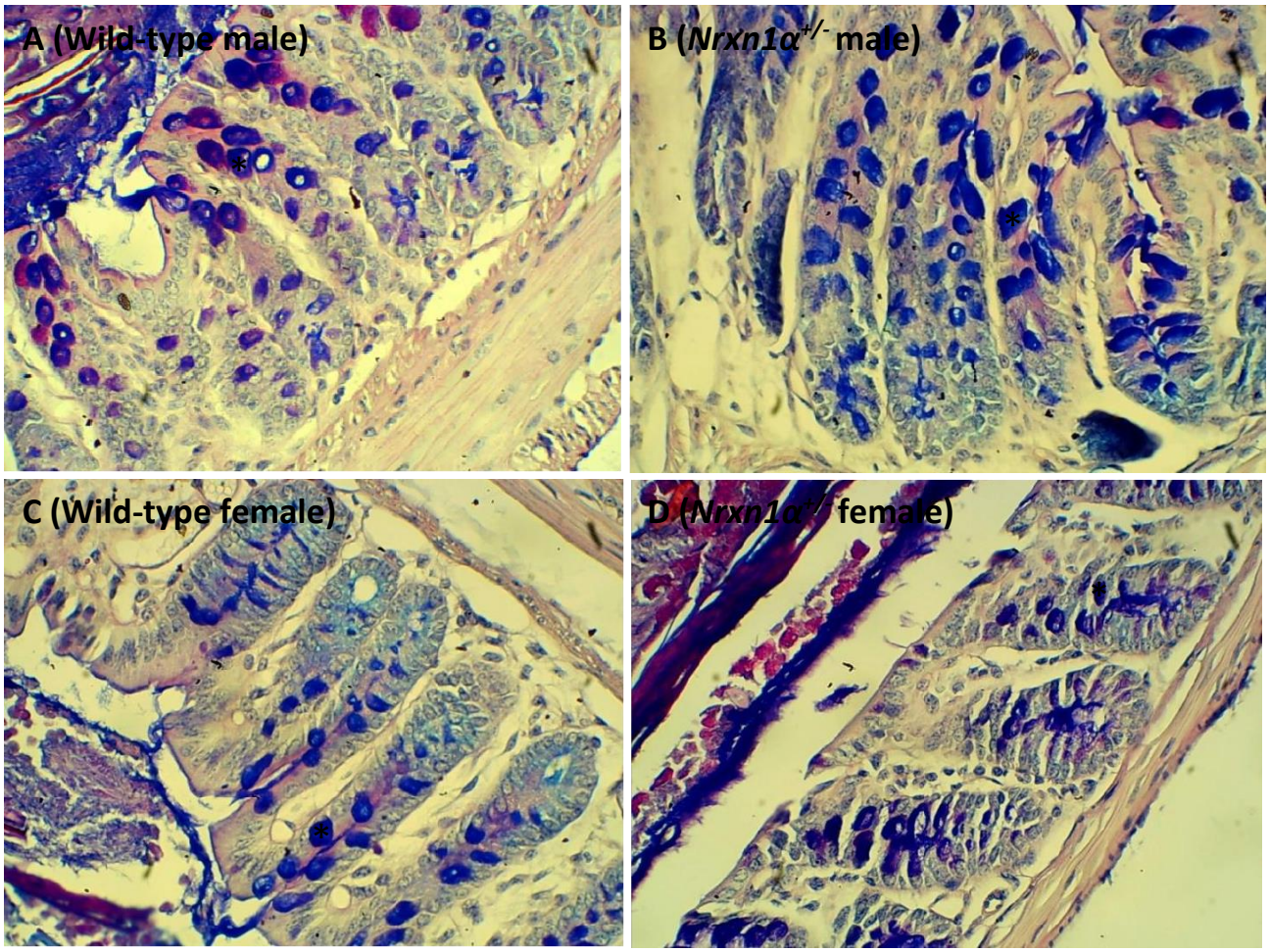


Figure 23: Representative PAS/AB stained images for goblet cell number in the colon. Images showing (a) a wild-type male, (b) a *Nrxn1α*$^{+/-}$ male, (c) a wild-type female, and (d) a *Nrxn1α*$^{+/-}$ female. These mice were all treated with saline. * indicates the presence of a goblet cell.

3.1.2.4 *Neurexin1α* heterozygosity has no significant effect on the size of goblet cells in the colon or caecum

In contrast to these observations on goblet cell number we found that *Nrxn1α* heterozygosity does not significantly impact on the size of the goblet cells in either the top layer ($p = 0.462$, $F_{(1,27)} = 0.558$) or bottom layer ($p = 0.396$, $F_{(1,27)} = 0.743$) of colon, or in the top ($p = 0.305$, $F_{(1,27)} = 1.093$) or bottom layer ($p = 0.111$, $F_{(1,27)} = 2.717$) of caecum when we analysed the mean size of goblet cells per 20 crypts in saline-only animals. No significant sex x genotype interactions were seen in the top layer ($p = 0.227$, $F_{(1,27)} = 1.530$, Figure 24a, Figure 24b) or bottom layer ($p = 0.182$, $F_{(1,27)} = 1.881$, Figure 24c, Figure 24d) of the colon, or in the top layer ($p = 0334$, $F_{(1,27)} =0.966$, Figure 25a, Figure 25b) or the bottom layer ($p = 0.150$, $F_{(1,27)} = 2.200$, Figure 25c, Figure 25d) of the caecum.

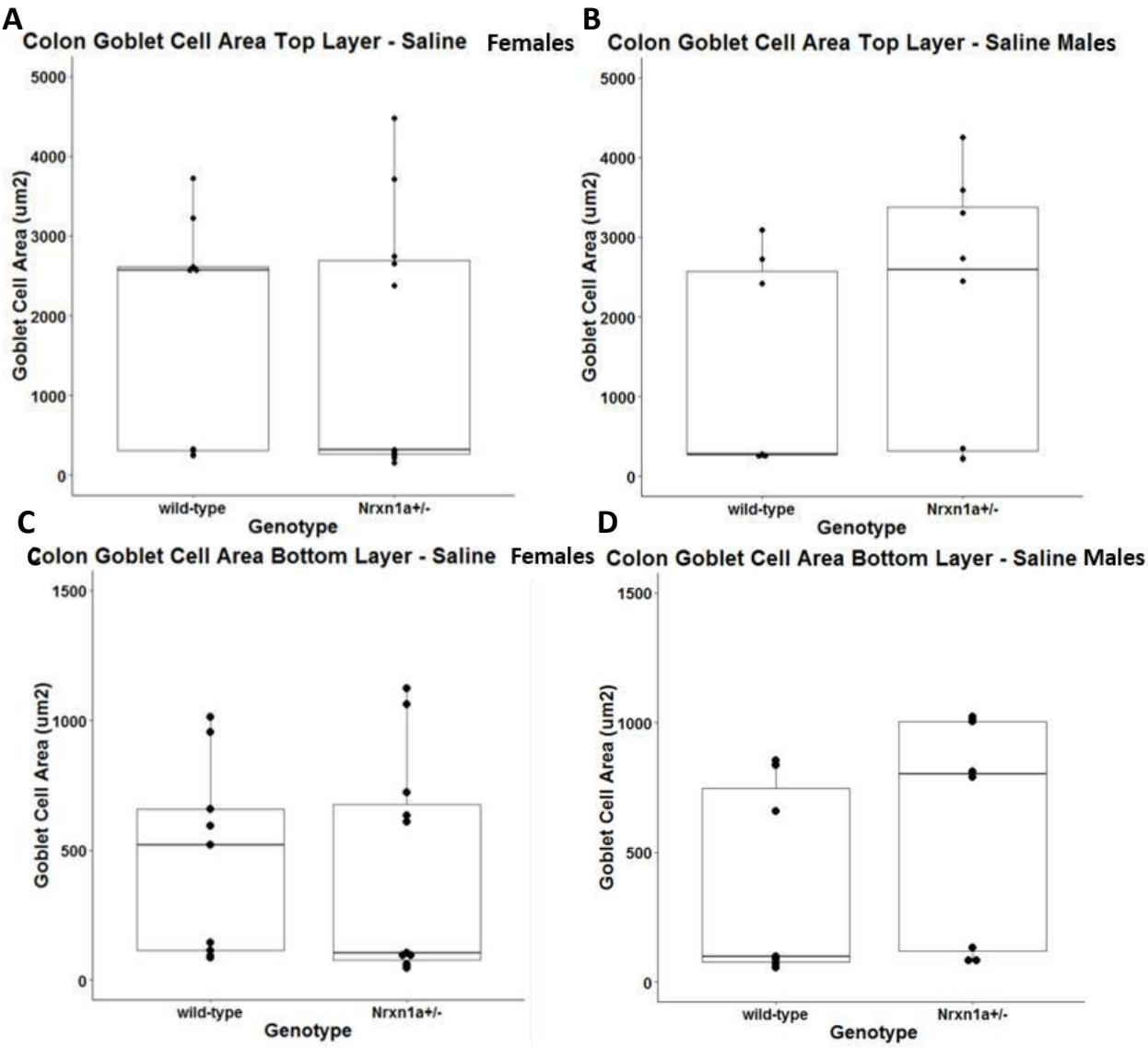


Figure 24: *Neurexin1α* heterozygosity has no significant effect on the size of goblet cells in the colon of saline-treated mice. (a) Colon goblet cell size in the top layer of wild-type and *Nrxn1α*$^{+/-}$ female crypts (wild-type females n = 7, *Nrxn1α*$^{+/-}$ females n = 9). (b) Colon goblet cell size in the top layer of wild-type and *Nrxn1α*$^{+/-}$ male crypts (wild-type males n = 6, *Nrxn1α*$^{+/-}$ males n = 7). (c) Colon goblet cell size in the bottom layer of wild-type and *Nrxn1α*$^{+/-}$ female crypts (wild-type females n = 8, *Nrxn1α*$^{+/-}$ females n = 10). Colon goblet cell size in the bottom layer of wild-type and *Nrxn1α*$^{+/-}$ male crypts (wild-type males n = 6, *Nrxn1α*$^{+/-}$ males n = 7). n-values include samples from both treatment groups as there was no significant treatment x genotype interactions.

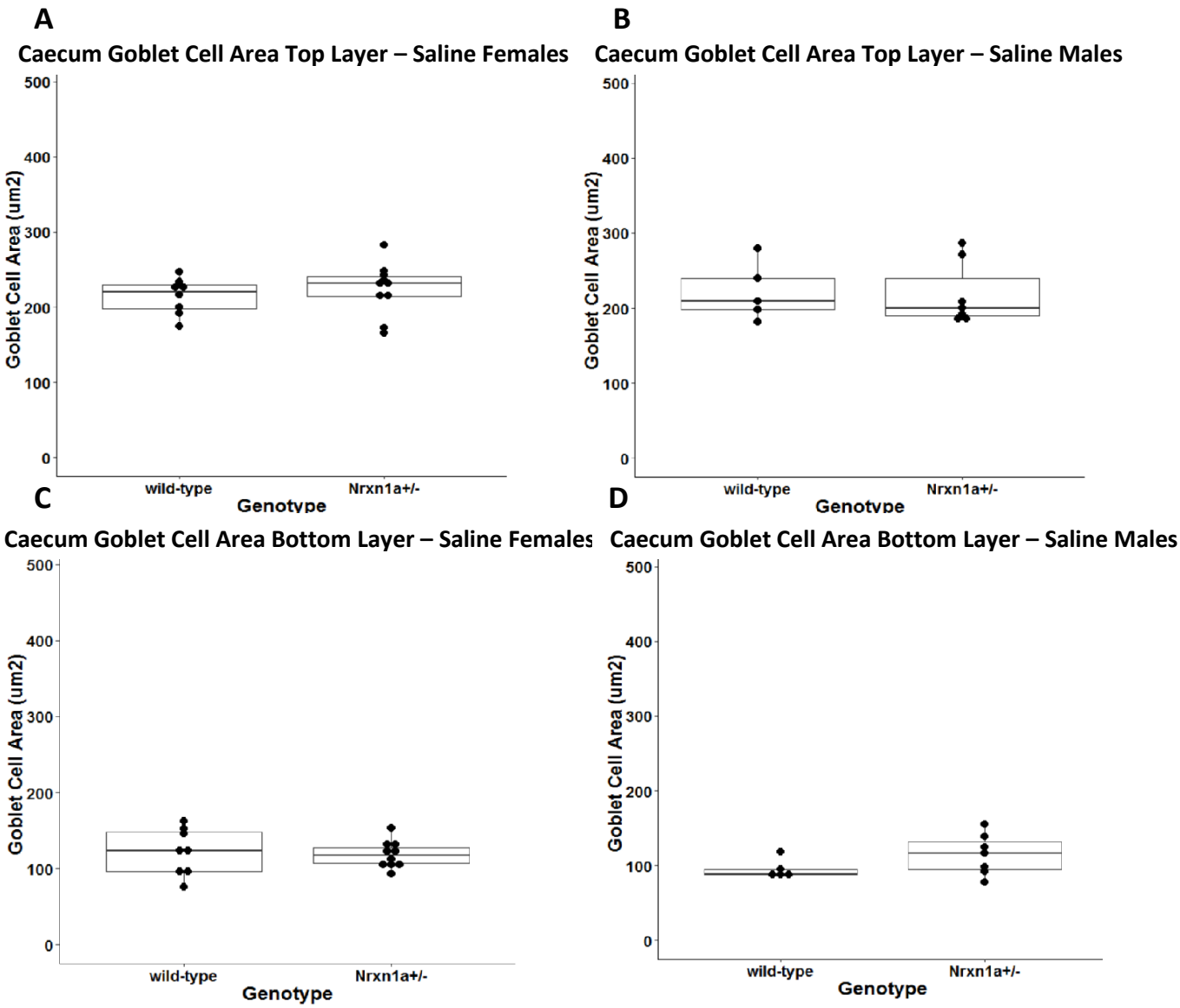


Figure 25: *Neurexin1α* heterozygosity has no significant effect on the size of goblet cells in the caecum of saline-treated mice. (a) Caecum goblet cell size in the top layer of wild-type and *Nrxn1α*$^{+/-}$ female crypts (wild-type females n = 8, *Nrxn1α*$^{+/-}$ females = 9). (b) Caecum goblet cell size in the top layer of wild-type and *Nrxn1α*$^{+/-}$ male crypts (wild-type males n = 5, *Nrxn1α*$^{+/-}$ males n = 7). (c) Caecum goblet cell size in the bottom layer of wild-type and *Nrxn1α*$^{+/-}$ female crypts (wild-type females n = 8, *Nrxn1α*$^{+/-}$ females n = 10). (d) Caecum goblet cell size in the bottom layer of wild-type and *Nrxn1α*$^{+/-}$ male crypts (wild-type males n = 5, *Nrxn1α*$^{+/-}$ males n = 7). n-values include samples from both treatment groups as there was no significant treatment x genotype interactions.

3.1.2.5 EEC number is significantly decreased in the caecum of *Neurexin1α+/-* female, but not male, mice

EEC's were analysed under the fluorescent microscope using anti-CgA and DAPI stains. The average total number of cells per 20 crypts were counted and compared between groups. We found an almost significant sex x genotype interaction for EEC cell number in the caecum ($p = 0.051$, $F_{(1,23)} = 4.448$). When the sexes were analysed independently using ANOVAs, we found that female *Nrxn1α+/-* mice showed significantly reduced EEC numbers in the caecum (genotype main effect, $p = 0.0273$, $F_{(1,23)} = 6.180$) while male mice did not ($p = 0.417$, $F_{(1,23)} = 0.734$), suggesting a sex-dependent genotype effect (Figure 26a and Figure 26b). Representative anti-CgA/DAPI images of EECs in the caecum, taken at x40 magnification, are shown in Figure 27.

By contrast we found no significant differences in the number of EECs counted in the colon. There were no significant differences in sex ($p = 0.964$, $F_{(1,22)} = 0.002$) or genotype ($p = 0.526$, $F_{(1,22)} = 0.415$). We also found no significant sex x genotype interactions in the colon ($p = 0.0629$, $F_{(1,22)} = 3.859$, Figure 26c, Figure 26d).

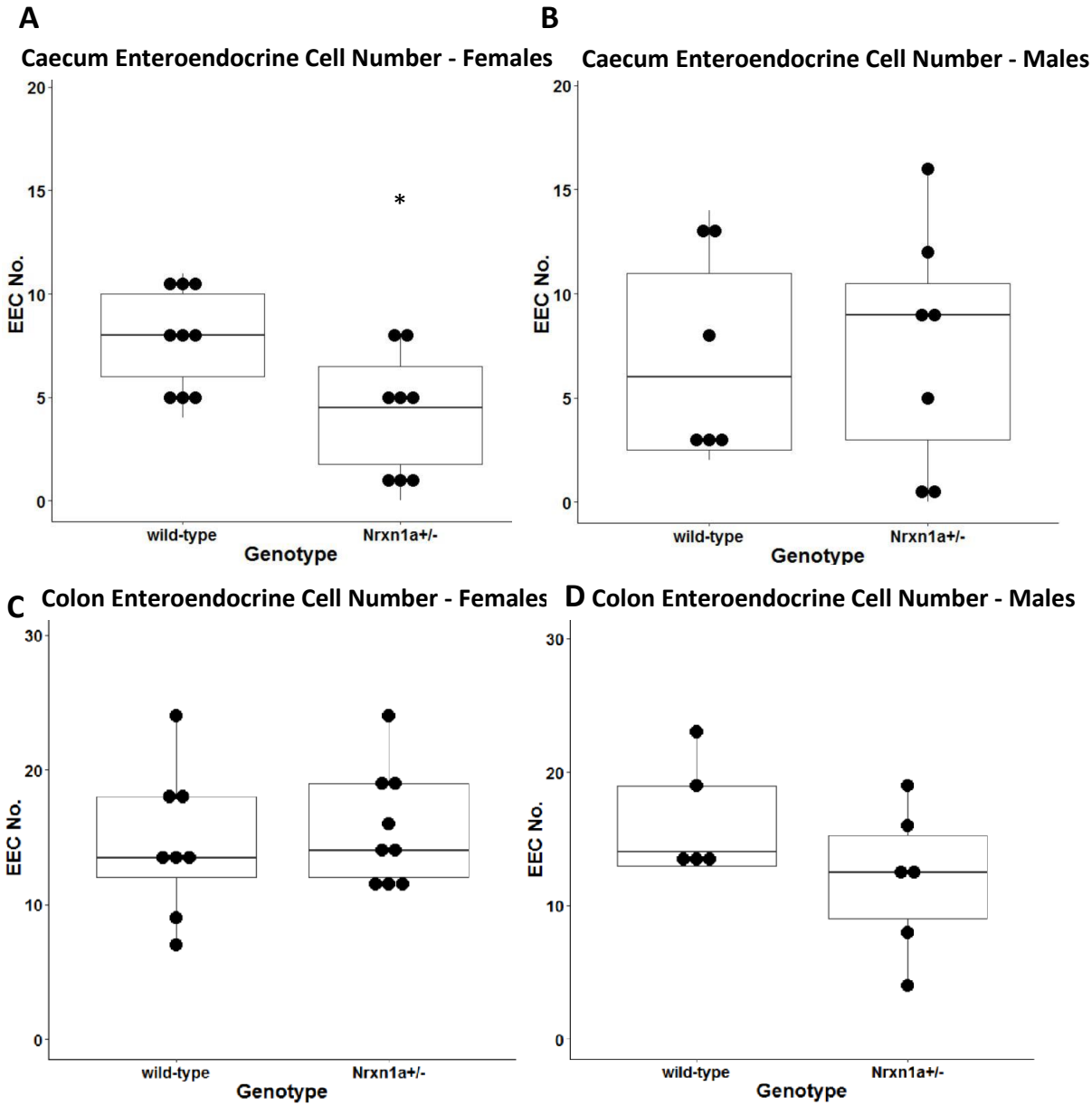


Figure 26: *Neurexin1α* heterozygosity decreases the number of EECs in the caecum of female, but not male, mice. (a) Caecum EEC cell number in wild-type and *Nrxn1α* $^{+/-}$ females (wild-type females n = 9, *Nrxn1α*$^{+/-}$ females n = 8). (b) Caecum EEC number in wild-type and *Nrxn1α* $^{+/-}$ males (wild type males n = 6, *Nrxn1α*$^{+/-}$ males n = 7). (c) Colon EEC number in wild-type and *Nrxn1α* $^{+/-}$ females (wild-type females n = 8, *Nrxn1α*$^{+/-}$ females n = 9). (d) Colon EEC number in wild-type and *Nrxn1α* $^{+/-}$ males (wild-type males n = 5, *Nrxn1α*$^{+/-}$ males = 6). n-values include samples from both treatment groups as there was no significant treatment x genotype interactions. *P<0.05 compared to relevant control (ANOVA).

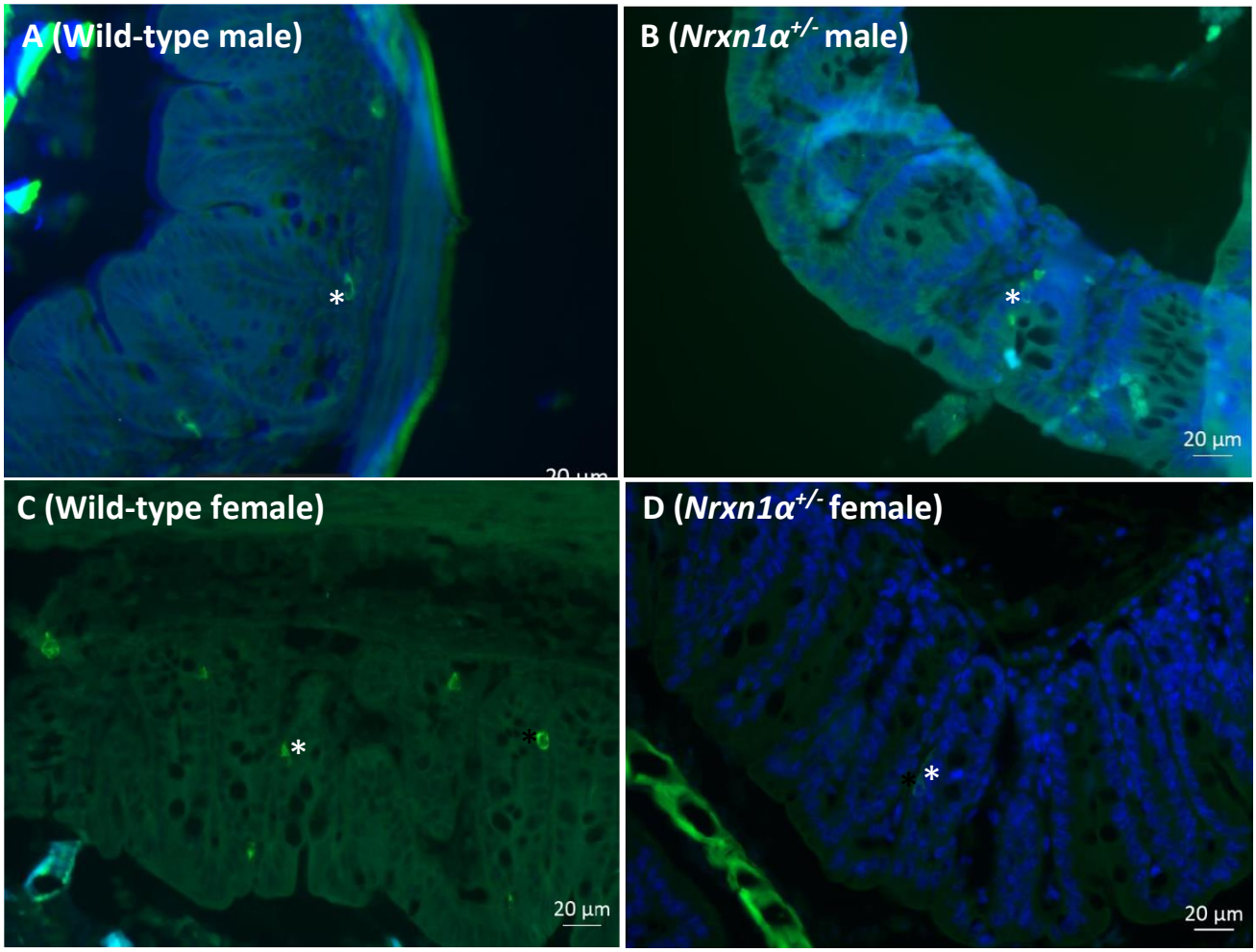


Figure 27: Representative anti-CgA/DAPI stained images for EECs in the caecum. Images showing a (a) wild-type male, (b) *Nrxn1α*$^{+/-}$male, (c) wild-type female, and (d) *Nrxn1α* $^{+/-}$ female. All mice shown were treated with saline. * indicates the presence of an EEC (bright green).

3.1.2.6 GLP-1 positive EEC cell number is significantly decreased in the colon of male, but not female, *Neurexin1α* heterozygous mice

Given our previous observations of altered EEC cell number in *Nrxn1α*$^{+/-}$ mice the number of EECs positive for the secretary product, GLP-1, was also measured to determine any differences between the genotypes. A significant sex x genotype interaction on EEC number was found in the colon ($p = 0.018$, $F_{(1,24)} = 6.436$). Post-hoc testing revealed that EEC cell number was significantly decreased in the colon of *Nrxn1α*$^{+/-}$ male ($p = 0.017$) but not female ($p = 0.137$) mice (Figure 28). Finally, there were no significant differences in the number of GLP-1 +ve cells in the caecum in terms of either sex ($p = 0.676$, $F_{(1,10)} = 0.179$) or genotype ($p = 0.242$, $F_{(1,21)} = 1.452$), and there was no evidence for a significant sex x genotype interaction in this region ($p = 0.758$, $F_{(1,21)} = 0.079$). Representative GLP-1/DAPI images, taken at x40 magnification, are shown in Figure 29.

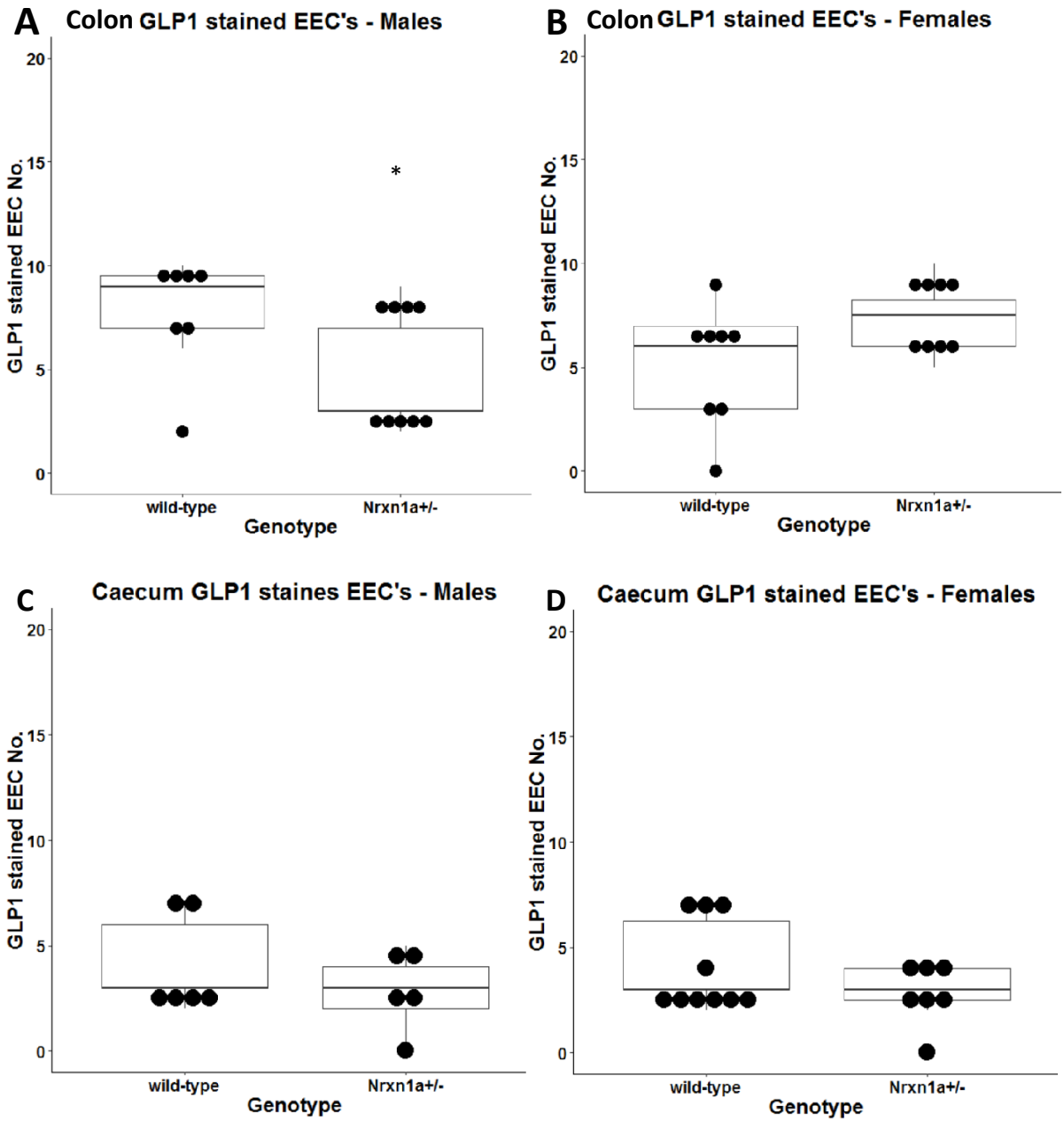


Figure 28: Neurexin1α heterozygosity decreases GLP-1 positive EEC number in the colon of male, but not female, mice. (a) Colon GLP-1 +ve EEC number in wild-type and *Nrxn1α* $^{+/-}$males (wild type males n = 7, *Nrxn1α*$^{+/-}$males n = 9. (b) Colon GLP-1 +ve EEC number in wild-type and *Nrxn1α*$^{+/-}$ females (wild-type females n = 8, *Nrxn1α*$^{+/-}$ females n = 8). (c) Caecum GLP-1 +ve EEC number in wild-type and *Nrxn1α*$^{+/-}$ males (wild-type males n = 6, *Nrxn1α*$^{+/-}$males n = 5). (d) Caecum GLP-1 +ve EEC cell number in wild-type and *Nrxn1α*$^{+/-}$ females (wild-type females n = 10, *Nrxn1α*$^{+/-}$ females n = 7). n-values include samples from both treatment groups as there was no significant treatment x genotype interactions. * P<0.01 compared to relevant control (Wilcoxon).

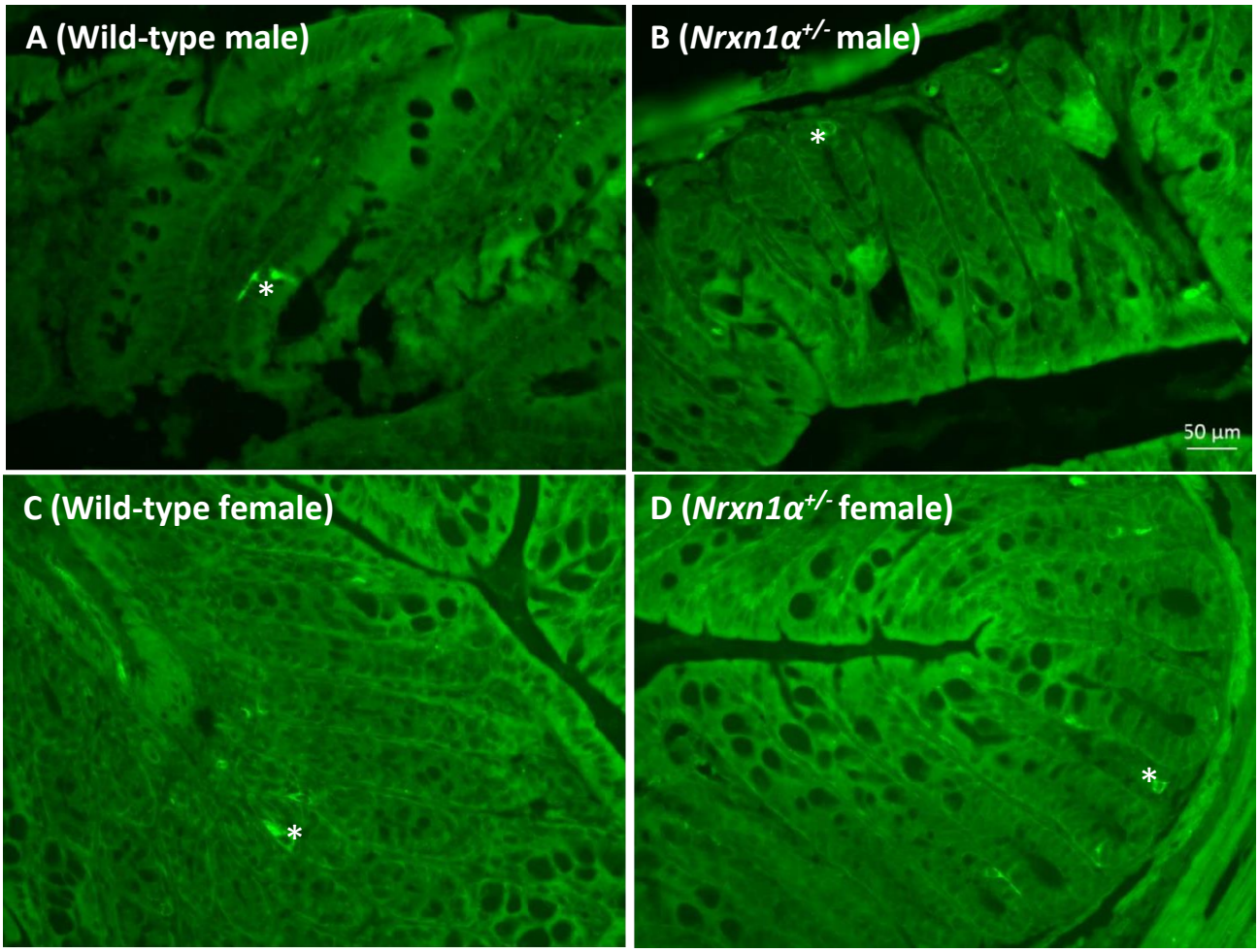


Figure 29: Representative images for GLP-1 +ve EECs in the colon. Images showing a (a) wild-type male, (b) *Nrxn1α*$^{+/-}$ male, (c) wild-type female, and (d) *Nrxn1α*$^{+/-}$ female. All mice were treated with saline*. * indicates the presence of a GLP-1 +ve EEC (bright green).

3.1.2.7 Summary: Impact of *Neurexin1α+/-* heterozygosity on the gut

The depth of the muscle layer was significantly decreased in the both the caecum and colon of mice heterozygous for *Nrxn1α*$^{+/-}$, and this effect was not significantly influenced by sex, which means there was no sex x genotype interaction here. In the caecum, there was a significant sex effect, which was independent of the genotype, where females had a significantly thicker muscle layer depth compared to males, which replicated the findings of our previous study.

In general, we found that the impact of *Nrxn1α*$^{+/-}$ on gut architecture was significantly more pronounced in females than in males. In this way, we found a sex x genotype interaction on the length of colon crypts; females with *Nrxn1α*$^{+/-}$ genotype had significantly smaller crypts in the colon in comparison to wild-type females. We also observed this same sex x genotype interaction for the number of mucus-secreting goblet cells counted. *Nrxn1α*$^{+/-}$ females had significantly fewer goblet cells than wild-type females while there was no evidence of this in males. The same sex-dependent genotype effect was observed for EEC count; fewer EECs were observed in *Nrxn1α*$^{+/-}$ females in the caecum in comparison to female wild-type mice, while there was no effect in males.

In contrast to these observations, GLP-1 +ve cell number was significantly decreased in the colon of *Nrxn1α*$^{+/-}$ males, but no significant changes were observed in *Nrxn1α*$^{+/-}$ females.

Overall, these data suggest that aspects of gut architecture dysfunction induced by *Nrxn1α*$^{+/-}$ are significantly influenced by sex, which predominantly occurs in female mice.

3.1.3 Impact of Liraglutide treatment

Liraglutide is a GLP-1 agonist which exerts GLP-1-like effects in the body which include the delay of gastric emptying and enhancing insulin secretion (Mehta et al, 2017). Liraglutide may exert antipsychotic-like effects in mice (Dixit et al, 2013). Previous work in our lab indicates that Liraglutide can restore some aspects of the cognitive dysfunction and brain dysfunction seen in *Neurexin1α* heterozygous mice (unpublished observations). Our data also suggest that GLP-1 secretion from the gut may be decreased in male *Nrxn1α*$^{+/-}$ mice (Figure 28a). Hence, we investigated the effects of Liraglutide treatment on the architecture of the gut to see if it could reverse the genotypic effects seen. Mice were treated with either Liraglutide or saline and had either wild-type or *Nrxn1α* $^{+/-}$ genotype. We did not see any evidence that Liraglutide reversed the impact of Nrxn1α heterozygosity on the gut. Therefore, here we focus on the significant effects of liraglutide on the gut. The majority of the effects were evident in both *Nrxn1α* $^{+/-}$ and wild-type mice.

3.1.3.1 Liraglutide treatment had no significant effects on the depth of the muscle layer

Treatment with Liraglutide did not significantly affect the depth of the muscle layer in the colon ($p = 0.558$, $F_{(1,30)} = 0.351$, Figure 30a) or in the caecum ($p = 0.783$, $F_{(1,25)} = 0.078$, Figure 30c). There was no impact of sex on the depth of the muscle layer in the colon ($p = 0.738$, $F_{(1,30)} = 0.114$, Figure 30b), although in the caecum females had a significantly thicker muscle layer depth than males ($p = 0.026$, $F_{(1,25)} = 5.575$, Figure 30d). There were no sex x treatment interactions in the colon ($p = 0.153$, $F_{(1,30)} = 2.149$) or in the caecum ($p = 0.633$, $F_{(1,25)} = 0.233$).

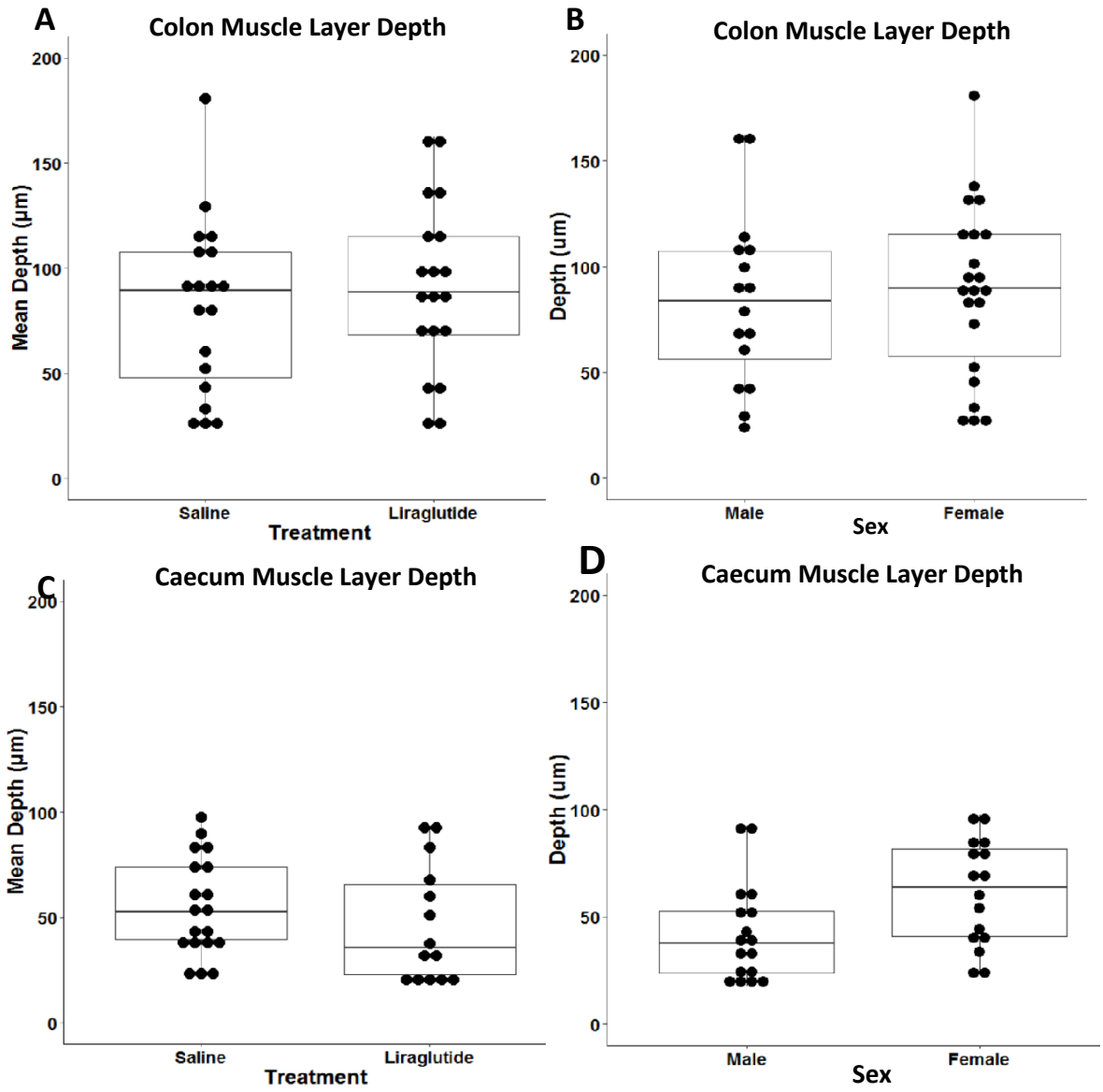


Figure 30: Liraglutide treatment has no significant effect on the depth of the muscle layer in the colon or caecum. In the caecum, there is a trend for females to have a thicker muscle layer than males. (a) Colon muscle layer depth in saline-treated and Liraglutide-treated mice (saline n = 19, Liraglutide n = 19, p = 0.558, F(1,30) = 0.351). (b) Colon muscle layer depth in males and females (males n = 16, females n = 22, p = 0.738, F(1,30) = 0.114). (c) Caecum muscle layer depth in saline-treated and Liraglutide-treated mice (saline n = 19, Liraglutide n = 14, p = 0.783, F(1,25) = 0.078). (d) Caecum muscle layer depth in males and females (males = n = 17, females n = 16, p = 0.026, F(1,25) = 5.575).

3.1.3.2 Liraglutide treatment had no significant effects on the length of crypts

The mean crypt length per 20 crypts in the colon or caecum were analysed using ImageJ software. We found that treatment with Liraglutide did not significantly affect the length of crypts in the colon ($p = 0.969$, $F_{(1,28)} = 0.002$, Figure 31a) or in the caecum ($p = 0.101$, $F_{(1,24)} = 2.902$, Figure 31c). There was no impact of sex on the length of colon crypts ($p = 0.971$, $F_{(1,28)} = 0.001$, Figure 31b), or on the caecum crypts ($p = 0.607$, $F_{(1,24)} = 0.272$, Figure 31d). There were no sex x treatment interactions in the colon ($p = 0.584$, $F_{(1,28)} = 0.308$) or in the caecum ($p = 0.308$, $F_{(1,24)} = 1.080$).

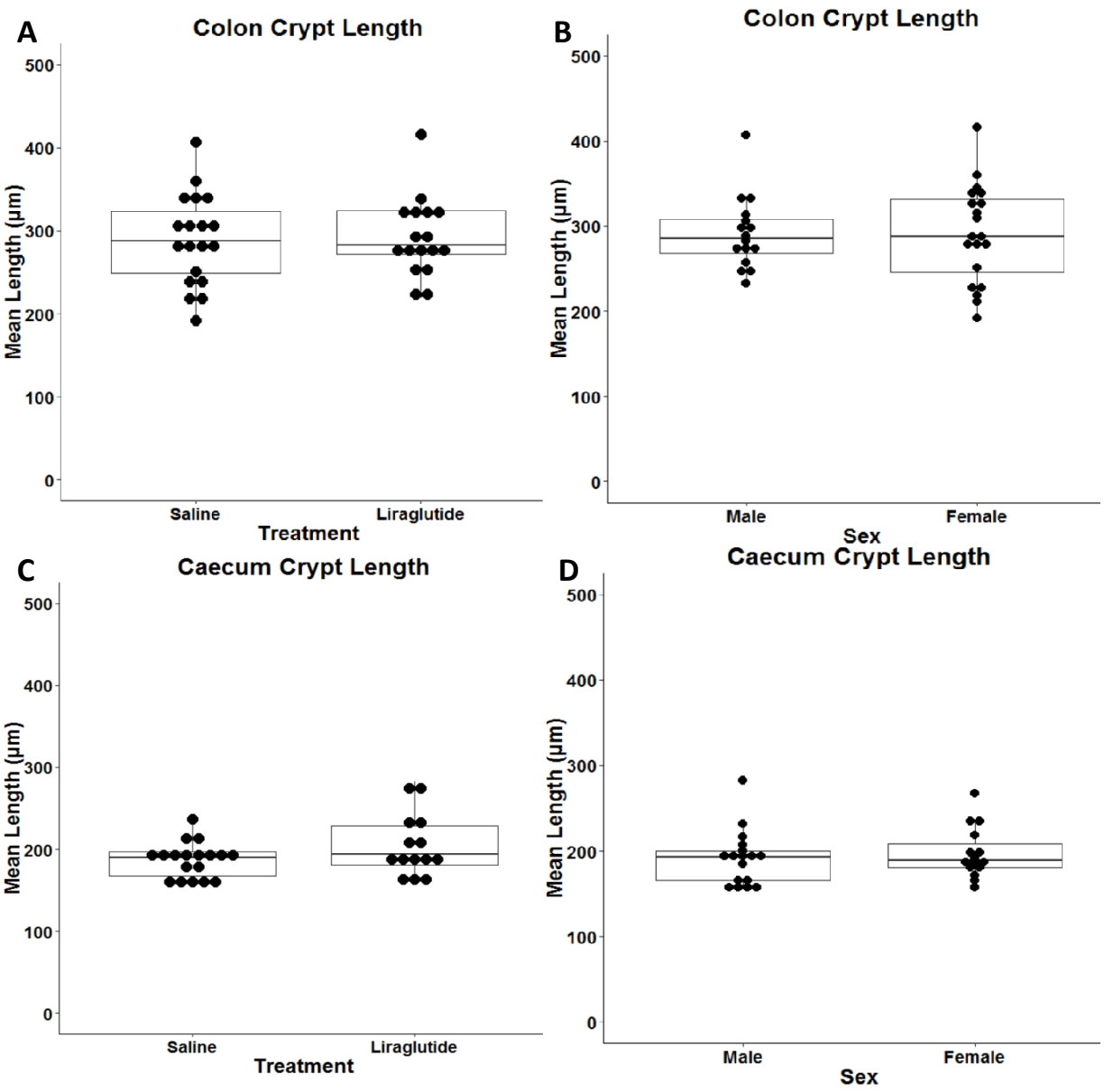


Figure 31: Liraglutide treatment has no effect on the length of crypts in the colon or caecum. (a) Colon crypt length in saline-treated and Liraglutide-treated mice (saline n = 19, Liraglutide n = 17, p = 0.969, $F_{(1,28)} = 0.002$). (b) Colon crypt length in males and females (males n = 16, females n = 20, p = 0.971, $F_{(1,28)} = 0.001$). (c) Caecum crypt length in saline-treated and Liraglutide-treated mice (saline n = 18, Liraglutide n = 14, p = 0.101, $F_{(1,24)} = 2.902$,). (d) Caecum crypt length in males and females (males n = 17, females n = 15, p = 0.607, $F_{(1,24)} = 0.272$).

3.1.3.3 Liraglutide had no significant effect on the number of goblet cells

Treatment with Liraglutide did not significantly affect the mean number of goblet cells counted per 20 crypts analysed in the colon ($p = 0.681$, $F_{(1,29)} = 0.173$, Figure 32a) or in the caecum ($p = 0.124$, $F_{(1,29)} = 2.504$, Figure 32c). There was no impact of sex on the mean number of goblet cells in the colon ($p = 0.160$, $F_{(1,29)} = 2.078$, Figure 32b), or in the caecum ($p = 0.643$, $F_{(1,29)} = 0.220$, Figure 32d). There were no sex x treatment interactions in the colon ($p = 0.975$, $F_{(1,29)} = 0.001$) or in the caecum ($p = 0.182$, $F_{(1,29)} = 1.870$).

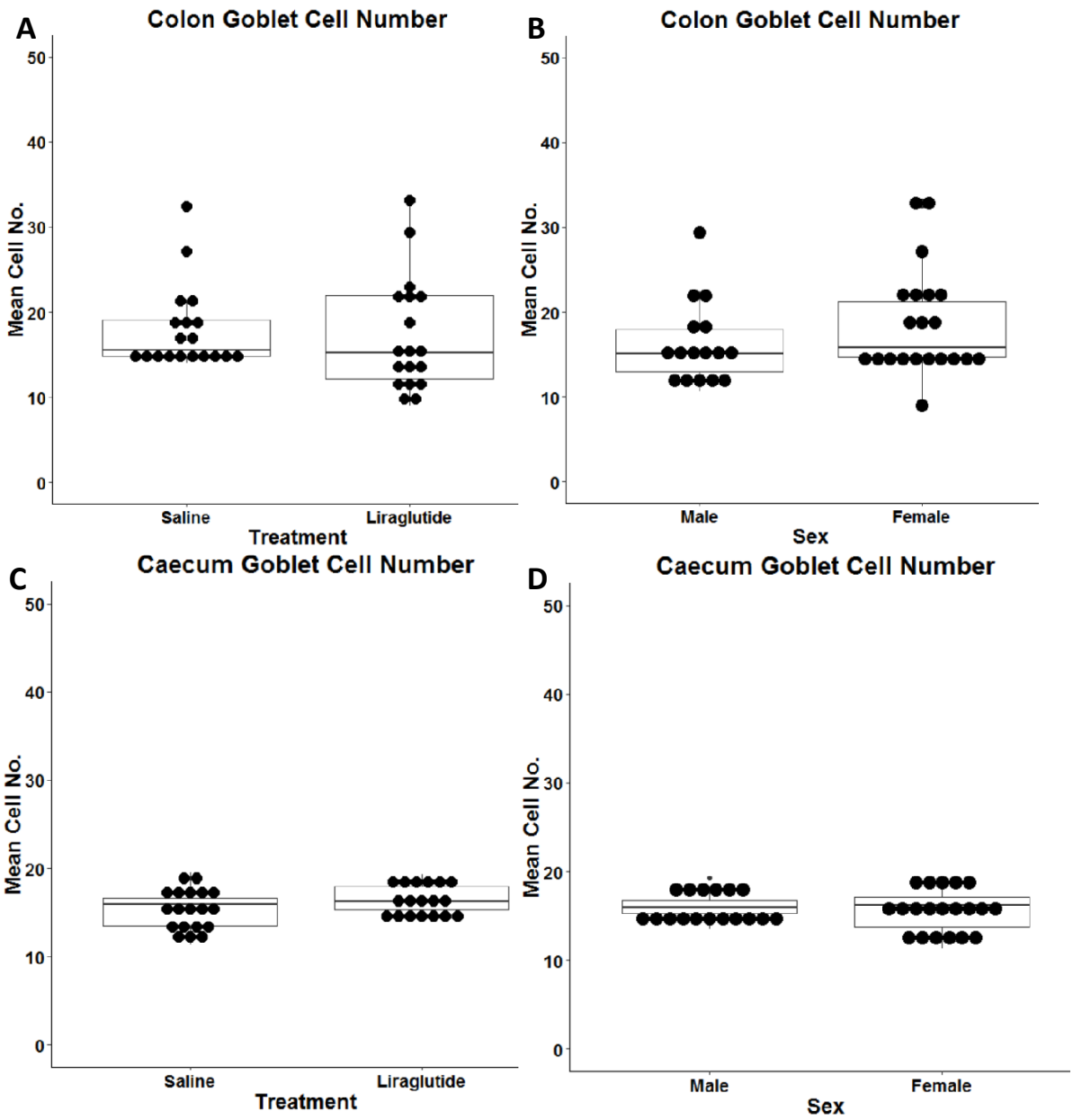


Figure 32: Liraglutide treatment had no significant effect on the number of goblet cells in the colon or caecum. (a) Colon goblet cell count in saline-treated and Liraglutide-treated mice (saline n = 19, Liraglutide n = 18, p = 0.681, $F_{(1,29)} = 0.173$). (b) Colon goblet cell count in males and females (males n = 16, females n = 21, p = 0.160, $F_{(1,29)} = 2.078$). (c) Caecum goblet cell count in saline-treated and Liraglutide-treated mice (saline n = 19, Liraglutide n = 18, p = 0.124, $F_{(1,29)} = 2.504$). (d) Caecum goblet cell count in males and females (males n = 17, females n = 20, p = 0.643, $F_{(1,29)} = 0.220$).

3.1.3.4 Liraglutide treatment decreases the size of goblet cells in the colon

Treatment with liraglutide significantly decreased the size of goblet cells in all areas of the colon. This was true in both the top layer ($p = 4.09e\text{-}07$, $F_{(1,27)} = 43.968$) and bottom layer ($p = 2.54e\text{-}07$, $F_{(1,27)} = 46.459$) of the colon crypts (Figure 33). These findings indicate there is a strong impact of liraglutide treatment on the size of goblet cells in the colon. We found no evidence that the impact of liraglutide on goblet cell area was significantly influenced by sex in the top (sex x treatment, $p = 0.174$, $F_{(1,27)} = 1.945$) and bottom (sex x treatment, $p = 0.243$, $F_{(1,27)} = 1.425$) layer of the colon (Figure 33). Representative PAS/AB images, taken at x40 magnification, are shown in Figure 34.

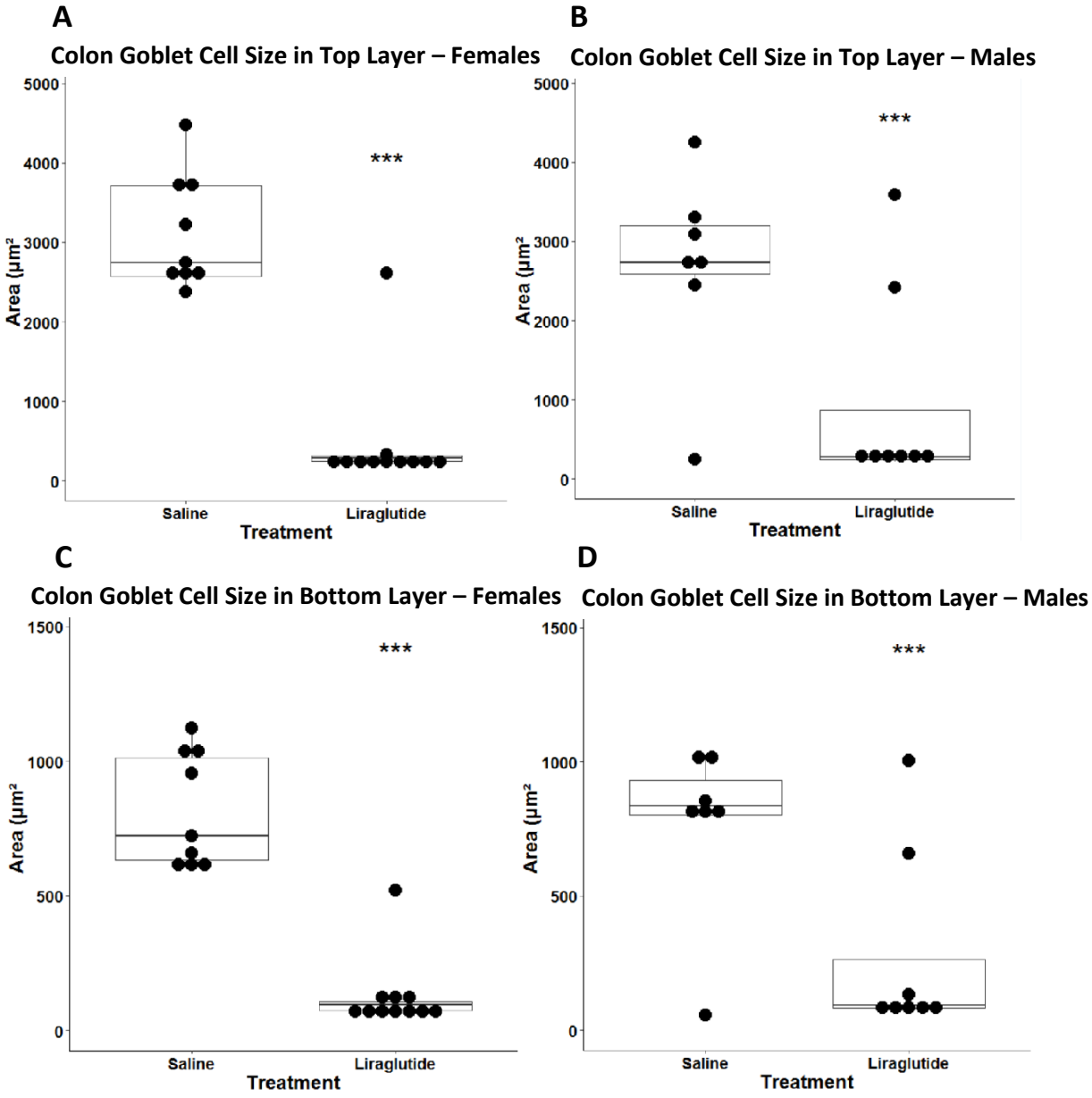


Figure 33: Liraglutide treatment decreases goblet cell size in the colon. (a) Colon goblet cell size (μm^2) in saline and Liraglutide-treated mice in the top layer of colon crypts in female mice (saline females n = 9, Liraglutide females n = 12). (b) Goblet cell size in saline and Liraglutide-treated mice in the top layer of colon crypts in male mice (saline males n = 7, Liraglutide males n = 8). (c) Goblet cell size in saline and Liraglutide-treated mice in the bottom layer of colon crypts in female mice (saline females n = 9, Liraglutide females n = 11). (d) Goblet cell size in saline and Liraglutide-treated mice in the bottom layer colon crypts in male mice. ***P<0.001 compared to relevant control (ANOVA). (saline males n = 7, Liraglutide males n = 8).

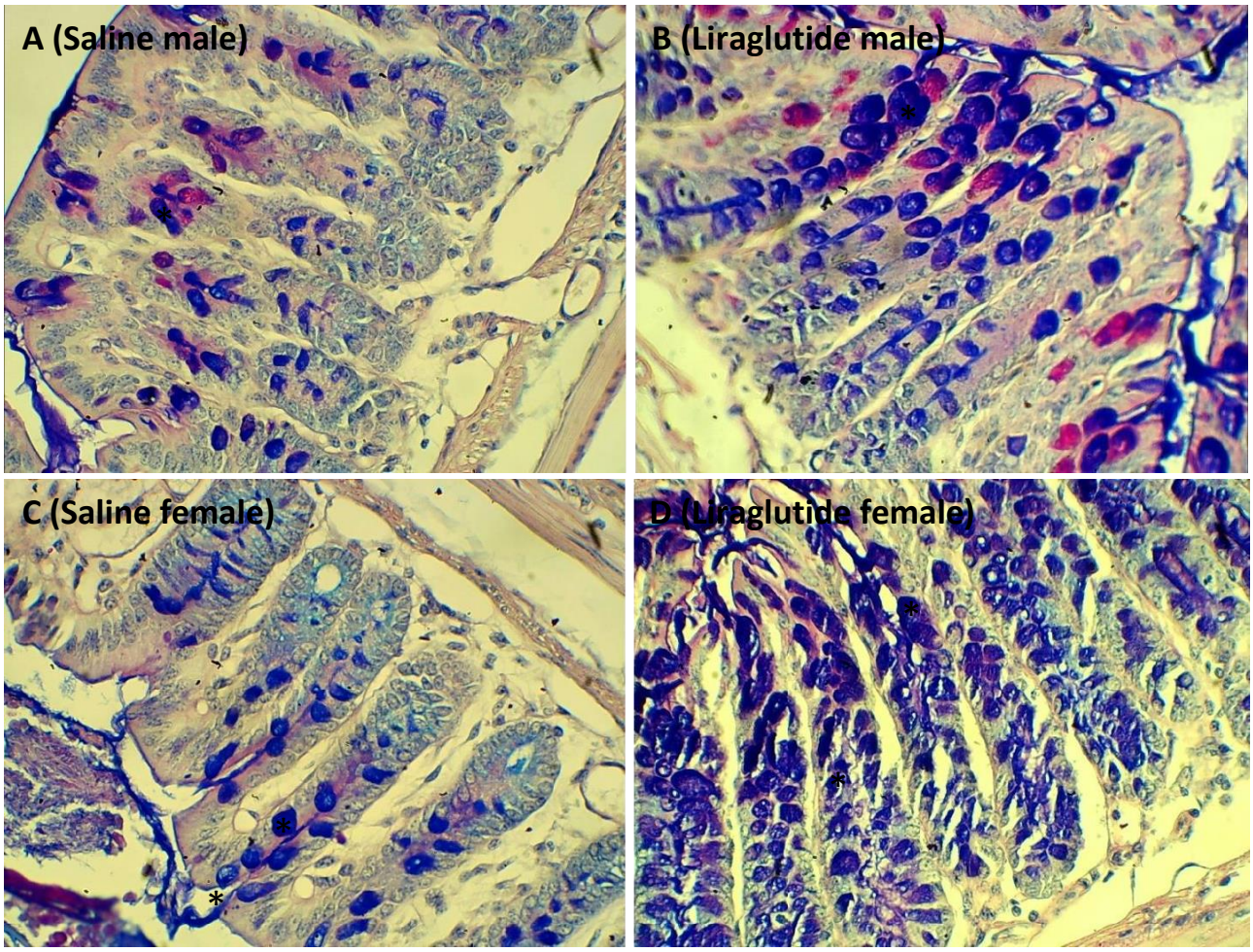


Figure 34: Representative PAS/AB stained images for goblet cells in the colon. Images showing (a) a saline-treated male, (b) a Liraglutide-treated male, (c) a saline-treated female, and (d) a Liraglutide-treated female. These mice were from the wild-type genotype. * represents the presence of a goblet cell.

3.1.3.5 Liraglutide treatment increases the size of goblet cells in the caecum in males, but not in females

Treatment with liraglutide significantly increased the size of goblet cells in all areas of the caecum in males only. This was true in both the top layer ($p = 0.004$, $F_{(1,27)} = 10.056$) and bottom layer ($p = 0.001$, $F_{(1,27)} = 12.556$) of the caecum crypts (Figure 35). These findings contradict what we observed in colonic goblet cells. In the caecum, we found evidence that the impact of liraglutide on goblet cell area was significantly influenced by sex in either the top (sex x treatment, $p= 0.004$, $F_{(1,27)} = 10.004$) or bottom (sex x treatment, $p = 0.003$, $F_{(1,27)} = 10.314$) layer of the caecum (Figure 35). Representative PAS/AB images, taken at x40 magnification, are shown in Figure 36.

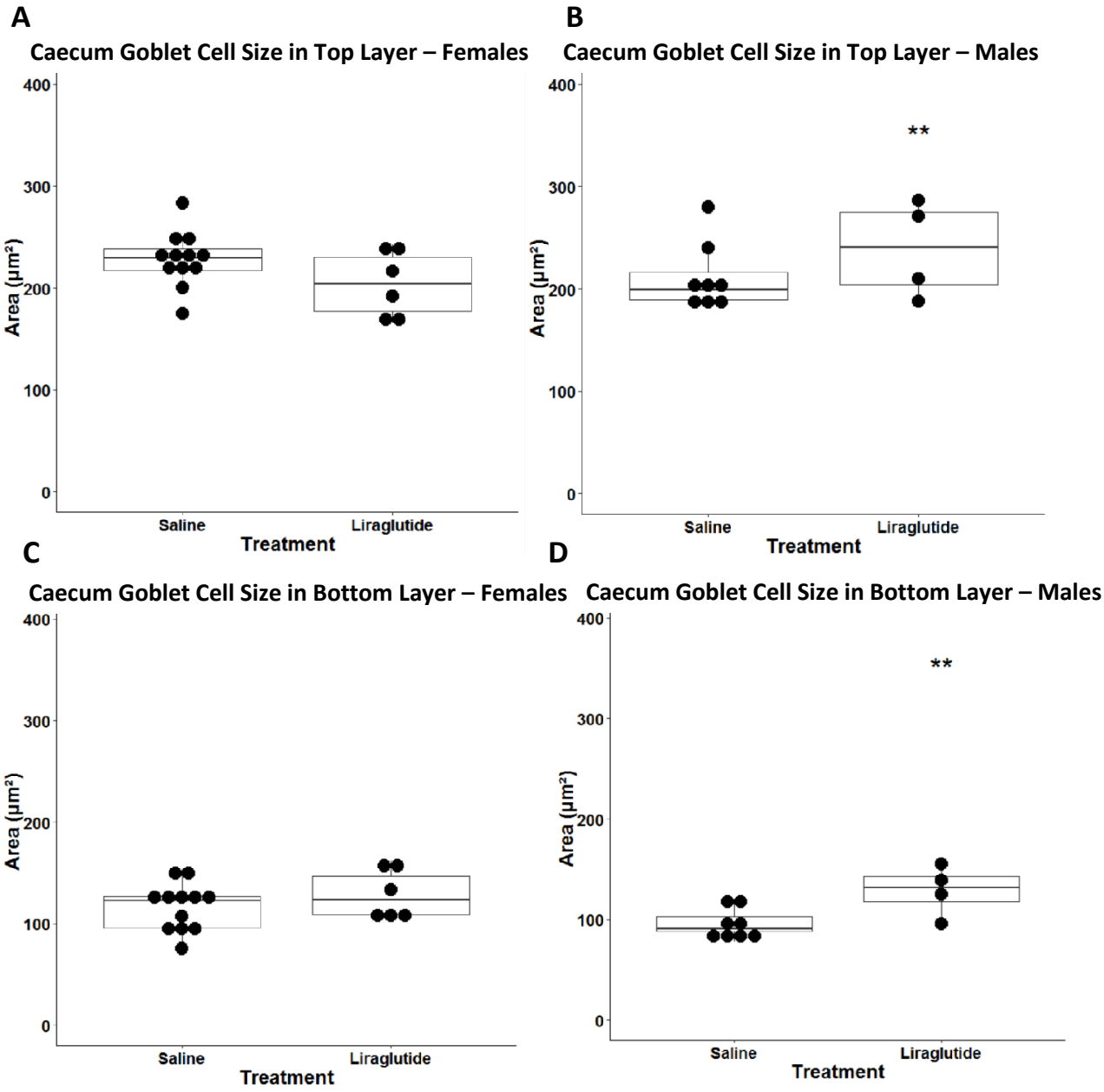


Figure 35: Liraglutide treatment increases goblet cell size in the colon in males. (a) Goblet cell size (μm^2) in saline and Liraglutide-treated mice in the top layer of female caecum crypts (saline females n = 12, Liraglutide females n = 6). (b) Goblet cell size in saline and Liraglutide-treated mice in the top layer of male caecum crypts (saline males n = 8, Liraglutide males n = 4). (c) Goblet cell size in saline and Liraglutide-treated mice in the bottom layer of female caecum crypts (saline females n = 12, Liraglutide females n = 6). (d) Goblet cell size in saline and Liraglutide-treated mice in the bottom layer of male caecum crypts (saline names n = 8, Liraglutide males n = 4).

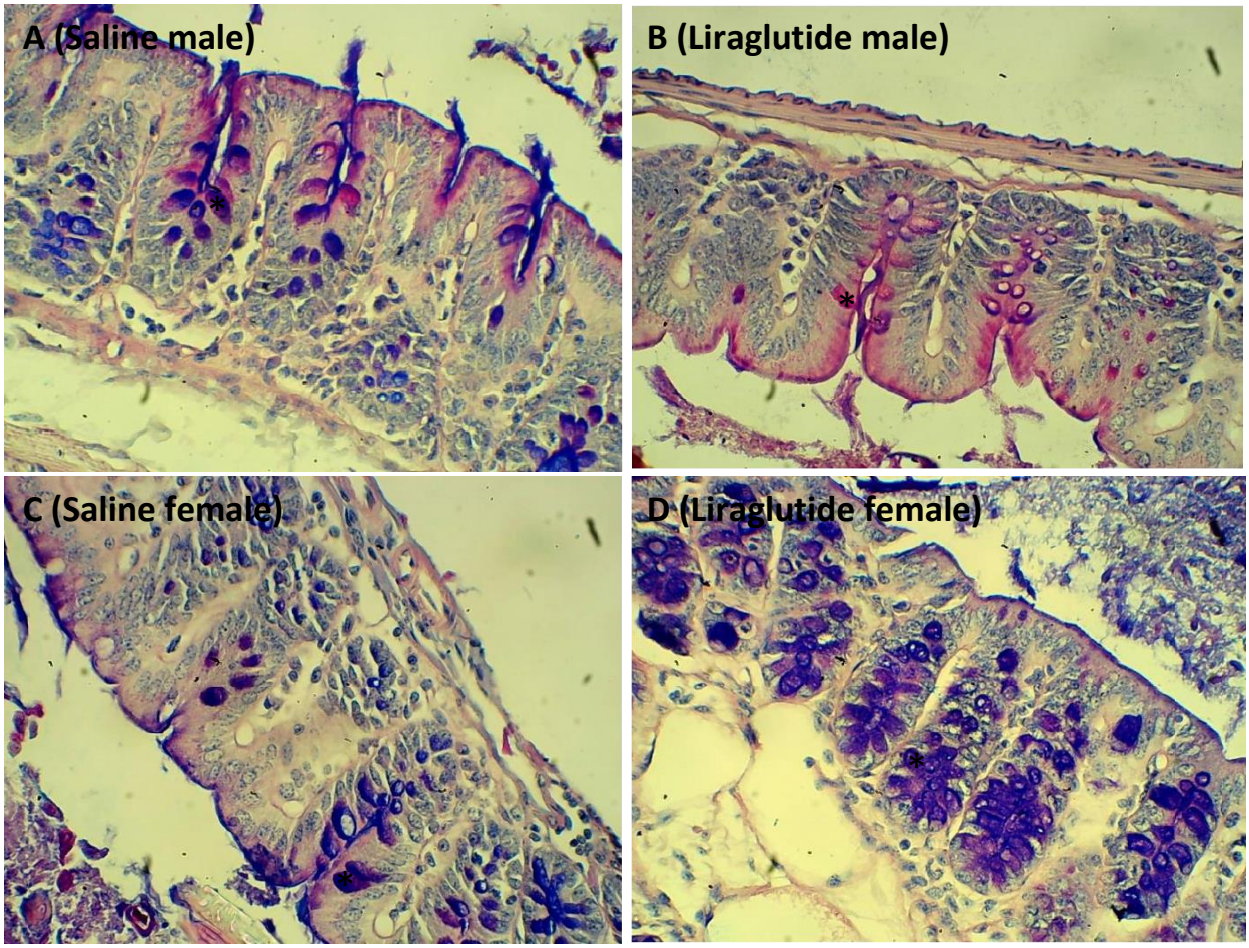


Figure 36: Representative PAS/AB stained images for goblet cells in the caecum. Images showing (a) a saline-treated male, (b) a Liraglutide-treated male, (c) a saline-treated female, and (d) a Liraglutide-treated female. These mice were from the wild-type genotype. * represents the presence of a goblet cell.

3.1.3.6 Liraglutide treatment selectively decreases EEC number in the caecum of *Neurexin1α* heterozygous male mice

We found evidence for a significant sex x treatment x genotype interaction on the number of EEC's in the caecum ($p = 0.002$, $F_{(1,23)} = 11.965$), therefore we analysed the data from each sex in two independent ANOVAs. In male mice we found evidence for a significant genotype x treatment interaction ($p = 0.012$, $F_{(1,10)} = 9.322$) and post-hoc testing confirmed the liraglutide treatment has a significant effect on EEC number in *Nrxn1α*$^{+/-}$ ($p = 0.036$, Tukey's HSD) but not wild-type ($p = 0.654$, Tukey's HSD) male mice. By contrast, we found no evidence for a significant genotype x treatment interaction in female mice ($p = 0.247$, $F_{(1,13)} = 1.469$). This suggests that liraglutide treatment significantly decreases the number of EECs in the caecum of *Nrxn1α* heterozygous male mice (Figure 37a). Representative CgA/DAPI stained images, taken at x40 magnification, are shown in Figure 38.

By contrast, in the colon there were no significant effects of Liraglutide treatment on EEC cell number ($p = 0.954$, $F_{(1,21)} = 0.003$), and there were no significant differences in sex ($p = 0.731$, $F_{(1,21)} = 0.122$). There were no significant sex x treatment interactions ($p = 0.637$, $F_{(1,21)} = 0.230$).

Furthermore, there were no significant changes to the quantity of EEC's which stained positive for GLP-1. Liraglutide treatment had no significant impact upon the GLP1 +ve EEC count in the colon ($p = 0.668$, $F_{(1,24)} = 0.188$) or in the caecum ($p = 0.243$, $F_{(1,21)} = 1.441$). There was no evidence of a sex x treatment interaction in the colon ($p = 0.705$, $F_{(1,24)} = 0.147$) or caecum ($p = 0.810$, $F_{(1,21)} = 0.059$). There was no evidence of a genotype x treatment interaction in the colon ($p = 0.664$, $F_{(1,24)} = 0.194$) or caecum ($p = 0.621$, $F_{(1,21)} = 0.252$).

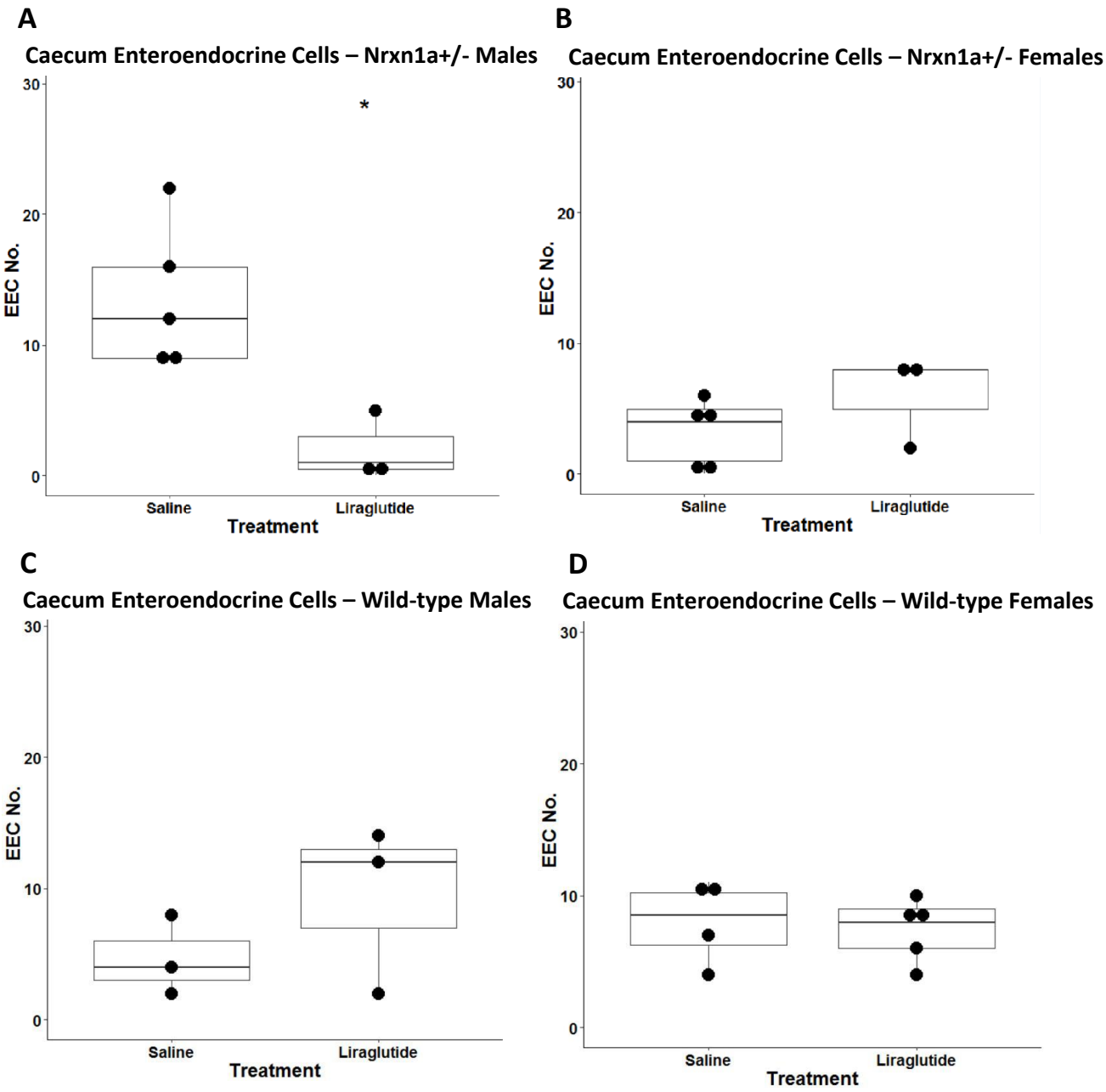


Figure 37: Liraglutide treatment decreases EEC cell numbers selectively in *Neurexin1α* heterozygous males in the caecum. (a) caecum total EEC number in heterozygous *Nrxn1α*$^{+/-}$ males treated with saline or Liraglutide (saline *Nrxn1α*$^{+/-}$ males n = 5, Liraglutide *Nrxn1α*$^{+/-}$ males n = 3). (b) caecum EEC number in heterozygous *Nrxn1α*$^{+/-}$ females treated with saline or Liraglutide (saline *Nrxn1α*$^{+/-}$ females n = 5, Liraglutide *Nrxn1α*$^{+/-}$ females n = 3). (c) caecum total EEC number in wild-type males treated with saline or Liraglutide (saline wild-type males n = 3, Liraglutide wild-type males n = 3). (d) caecum total EEC number in wild-type females treated with saline or Liraglutide (saline wild-type females n = 4, Liraglutide wild-type females n = 5). *P<0.05 compared to saline control (Tukey's HSD).

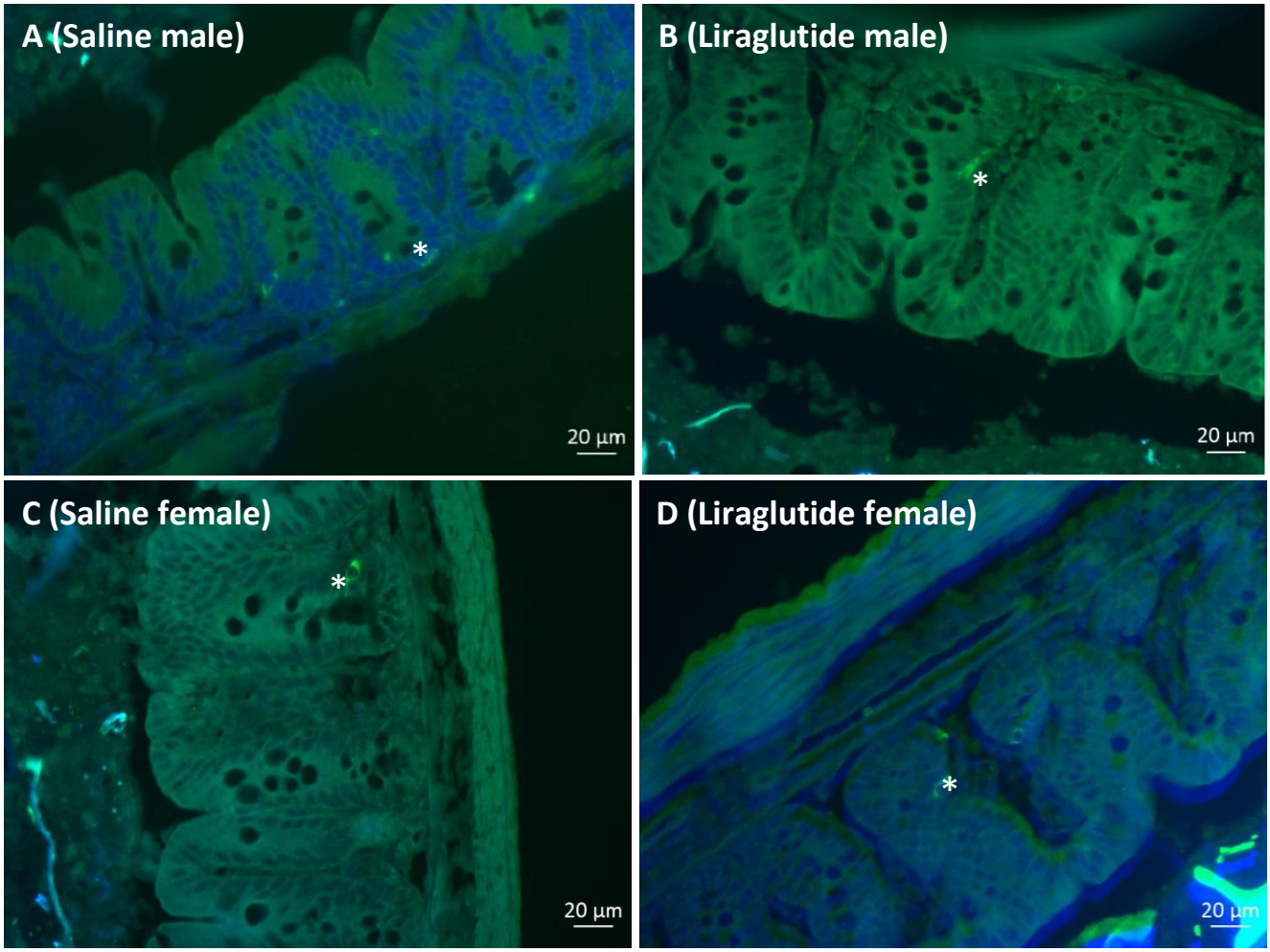


Figure 38: Representative CgA/DAPI stained images for EECs in the caecum. Images showing (a) a saline-treated male, (b) a Liraglutide-treated male, (c) a saline-treated female, and (d) a Liraglutide-treated female. All mice were from the *Nrxn1α*$^{+/-}$ genotype. * represents the presence of an EEC (bright green).

3.1.3.7 Summary: Impact of Liraglutide treatment

Liraglutide treatment significantly decreased the area of mucus-secreting goblet cells within the colon in animals of both sexes. However, in the caecum Liraglutide-treatment caused goblet cell size to significantly increase, and this effect was specific to males. There were no significant changes to the size of goblet cells in the caecum of female mice when treated with Liraglutide. These contrasting data indicate Liraglutide influences goblet cell size which is location and sex specific. It is possible that mucus-secretion in these goblet cells may be altered, however this was not investigated. We found no significant changes to the depth of the muscle layer in the colon or in the caecum in response to liraglutide treatment. Liraglutide treatment also had no significant effect on the mean number of goblet cells, or on the length of crypts in the colon and caecum.

Liraglutide treatment selectively decreases EEC number in the caecum of *Nrxn1α* heterozygous male mice (Figure 37a), but this effect was not seen in females (Figure 37b). This suggests Liraglutide treatment in the caecum is genotype and sex specific. Liraglutide treatment had no significant effect on EEC count in the colon. We also investigated the impact of Liraglutide treatment on EECs which stain positively for GLP-1 in the colon and caecum. Liraglutide is a GLP-1 agonist, hence may alter EEC function. We found no evidence that liraglutide significantly impacted on GLP-1 +ve cell number in either the colon or the caecum.

3.2 Gut Microbiome

We used DGGE analysis to determine the bacterial diversity within our samples from the experimental groups. DGGE separates 16s rDNA fragments based on their melting points through a polyacrylamide gel containing a liner gradient of DNA denaturants. A representative DGGE gel image taken from our PCP and saline treated mice is shown in Figure 39. Each lane corresponds to a DNA sample. The bands produced during electrophoresis represent a particular species of bacteria. Hence, if more bands are present there is increased bacterial diversity.

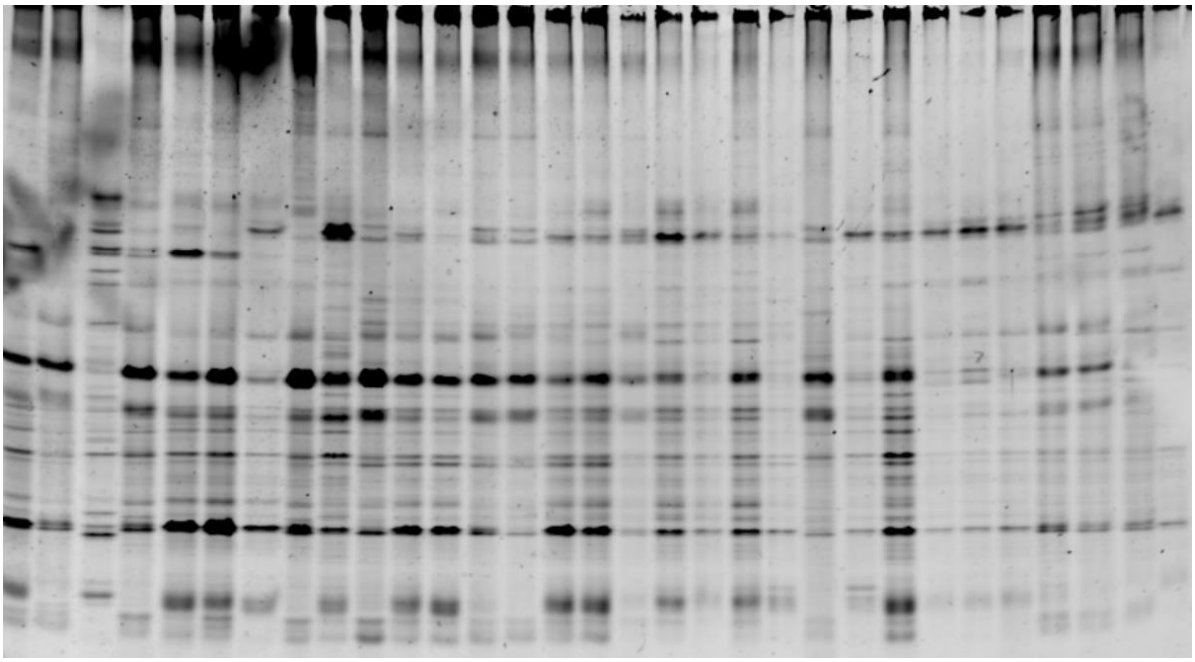


Figure 39: Representative DGGE gel image. A higher number of bands represents increased bacterial diversity within a sample.

3.2.1 Impact of PCP treatment and Sex, on the Number of Bacterial Species from DGGE band count analysis

We determined the number of bands between sample groups from DGGE analysis to determine any significant differences in the abundance of bacterial species. We observed no significant effects of PCP treatment on the number of bands between animals. However, there is likely a possible trend for a difference in bacterial species number in the colon lumen samples between male and female mice. A few outliers were removed from these analysis as they significantly differed from our general findings, potentially due to the lights DGGE bands seen for these samples. Thus 2 animals were removed from the caecum mucus, 2 from the colon lumen, and 6 from the colon mucus samples.

3.2.1.1 PCP treatment had no significant effect on number of bacterial species in the colon or caecum

Treatment with PCP caused no significant changes to the number of bands in the colon lumen ($p = 0.6688$, $F_{(1,9)} = 0.196$, Figure 40a) or to the colon mucus layer ($p = 0.400$, $F_{(1,10)} = 0.771$, Figure 40b). Furthermore, PCP also caused no significant changes to the number of bands in the caecum lumen ($p = 0.949$, $F_{(1,12)} = 0.004$, Figure 40c) or in the caecum mucus layer ($p = 0.888$, $F_{(1,10)} = 0.021$, Figure 40d), when compared to saline-treated mice. Furthermore, there were no significant interactions between sex and PCP treatment in the colon lumen ($p = 0.4479$, $F_{(1,9)} = 0.630$) and mucus layers ($p = 0.579$, $F_{(1,10)} = 0.329$), nor in the caecum lumen ($p = 0.139$, $F_{(1,12)} = 1.082$) and caecum mucus layer ($p = 0.586$, $F_{(1,10)} = 0.317$).

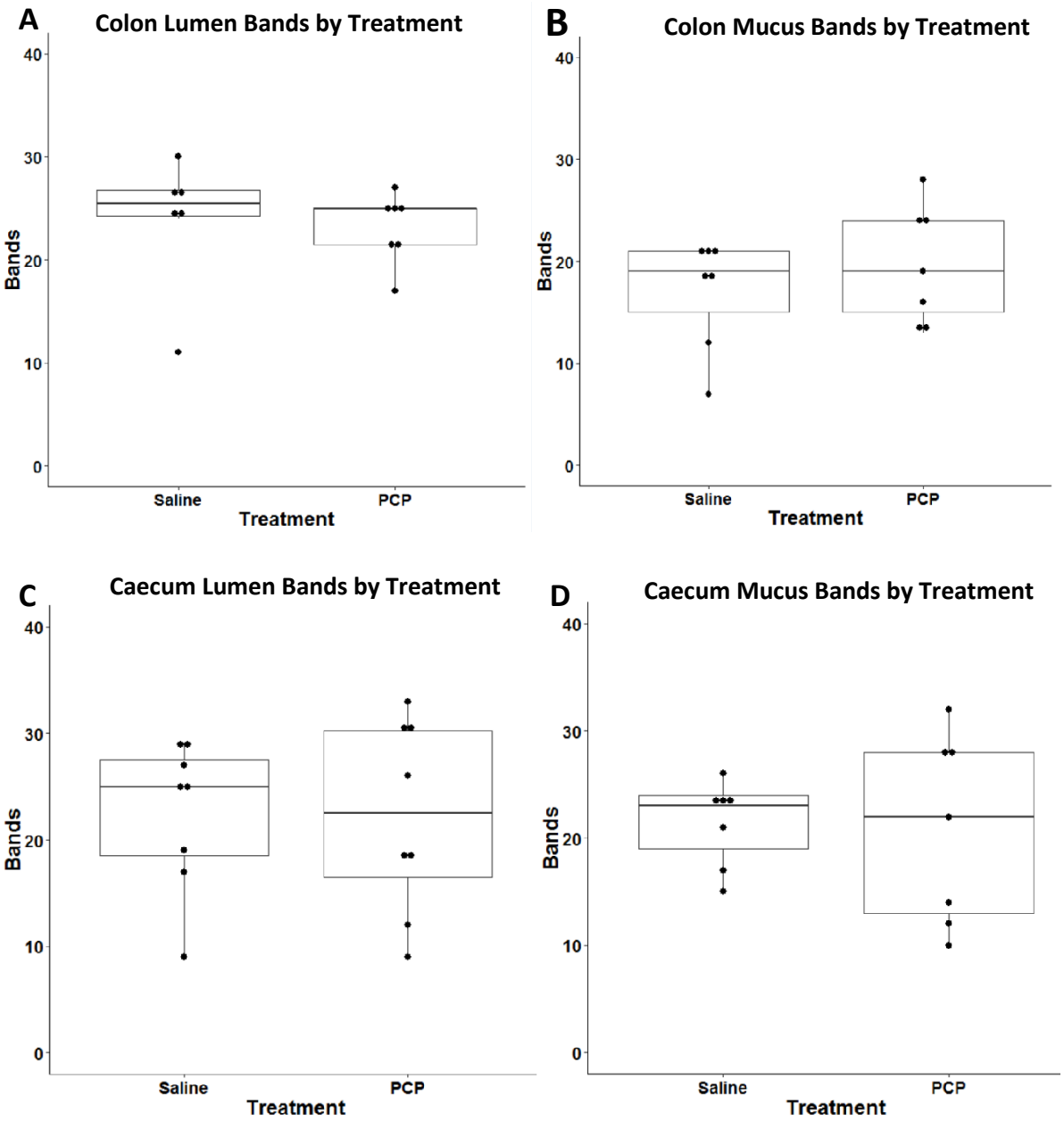


Figure 40: PCP had no significant effect on the number of bacterial species counted. Data shown as DGGE band counts in the (a) Colon lumen layer (saline n = 6, PCP n = 7, p = 0.6688, $F_{(1,9)} = 0.196$). (b) Colon mucus layer (saline n = 7, PCP n = 7, p = 0.400, $F_{(1,10)} = 0.771$). (c) Caecum lumen layer (saline n = 8, PCP n = 8, p = 0.949, $F_{(1,12)} = 0.004$). (d) Caecum mucus layer (saline n = 7, PCP n = 7, p = 0.888, $F_{(1,10)} = 0.021$).

3.2.1.2 In the colon lumen males have a significantly higher number of bacterial species, as shown by DGGE band counts, than females

In the colon lumen, males generally had more bands than females ($p = 0.0519$, $F_{(1,9)} = 5.014$, Figure 41a), although there were no significant differences in the number of bands in the colon mucus layer between the sexes ($p = 0.336$, $F_{(1,10)} = 0.897$, Figure 41b). In the caecum, the number of bands were similar between both sexes in the lumen ($p = 0.145$, $F_{(1,12)} = 2.436$, Figure 41c) but in the mucus layer there is a trend for an increased number of species in males than in females ($p = 0.823$, $F_{(1,10)} = 0.053$, Figure 41d).

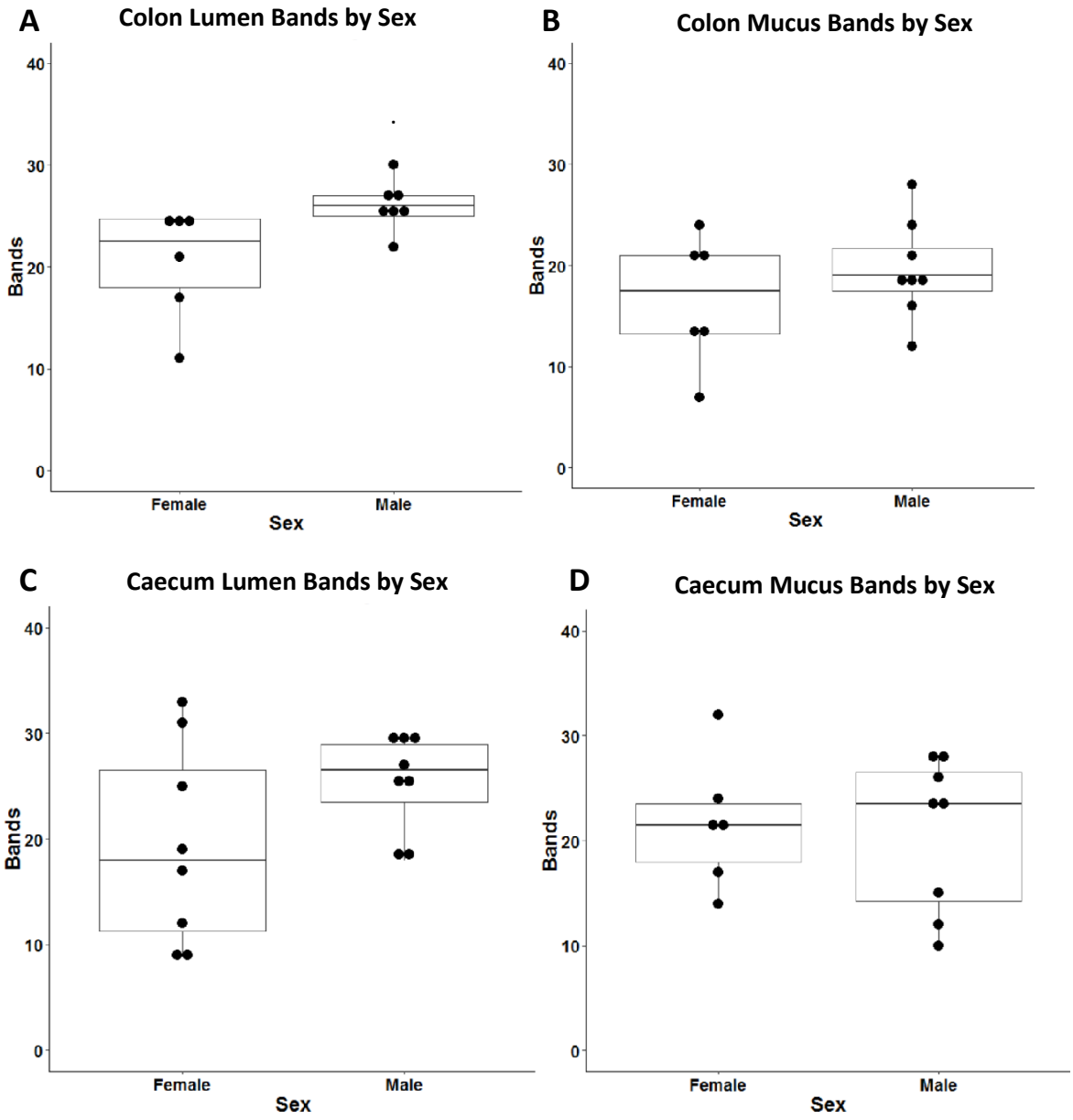


Figure 41: In the colon lumen males have a significantly higher number of bacterial species than females. Data shown as DGGE band counts in the (a) colon lumen layer (females n=6, males n=7, p=0.0519, $F_{(1,9)} = 5.014$), (b) colon mucus layer (females n = 6, males n = 8p=0.366, $F_{(1,10)} = 0.897$), (c) caecum lumen layer (females n=8, males n=8), (d) caecum mucus layer (females n = 6, males n = 8, p = 0.823, $F_{(1,10)} = 0.053$).

3.2.1.3 Sex does not significantly influence the impact of PCP on DGGE band count

There were no sex x treatment interactions on DGGE band count in the colon lumen (treatment x sex, $p = 0.4479$, $F_{(1,9)} = 0.630$), colon mucus (treatment x sex, $p = 0.579$, $F_{(1,10)} = 0.329$), caecum lumen (treatment x sex, $p = 0.319$, $F_{(1,12)} = 1.082$), or caecum mucus (treatment x sex, $p = 0.586$, $F_{(1,10)} = 0.317$).

3.2.2 Impact of *Neurexin1α*$^{+/-}$ heterozygosity, Liraglutide and Sex on the Number of Bacterial Species from DGGE band count analysis

Neurexin1α heterozygosity (*Nrxn1α*$^{+/-}$) had no significant effect on the abundance of bacterial species counted. Liraglutide treatment caused a higher number of bands in the caecum lumen and there was a trend for more bands in the caecum mucus layer. There is a higher number of bands counted in females than males in the colon lumen and possibly in the colon mucus layer. We saw no significant interactions between genotype and sex, genotype and treatment, or treatment and sex.

3.2.2.1 Impact of Neurexin1a heterozygosity

***Neurexin1α* heterozygosity had no significant effect on the number of bacterial species in the colon or caecum**

Nrxn1α heterozygosity had no effect on the number of DGGE bands counted in the colon lumen ($p = 0.9802$, $F_{(1,26)} = 0.001$, Figure 42a) or mucus layer ($p = 0.7912$, $F_{(1,18)} = 0.072$, Figure 42b) suggesting no differences in bacterial species when compared to wild-type mice. These effects were replicated in the caecum, where *Nrxn1α* heterozygosity also had no effect on the number of bands counted in the lumen ($p = 0.1265$, $F_{(1,28)} = 2.480$, Figure 42c) or in the mucus layer ($p = 0.7338$, $F_{(1,26)} = 0.118$, Figure 42d).

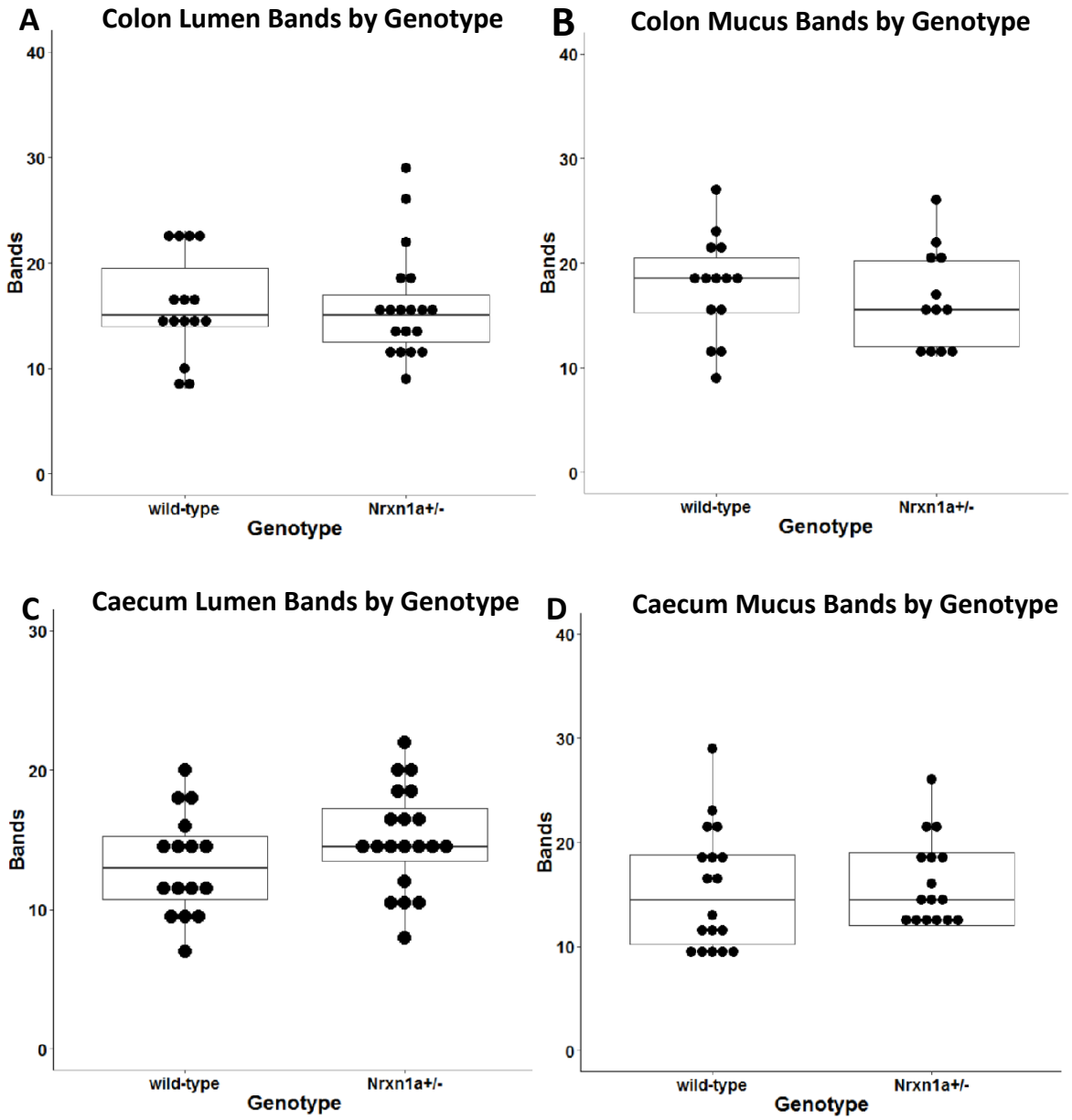


Figure 42: *Neurexin1α* heterozygosity had no significant effect on the number of bacterial species counted in the colon or caecum. Data shown as DGGE band counts in the (a) colon lumen (wild-type n = 15, *Nrxn1α* $^{+/-}$ n = 17, p = 0.9802, $F_{(1,26)}$ = 0.001), (b) colon mucus (wild-type n = 14, *Nrxn1α* $^{+/-}$ n = 12, p = 0.7912, $F_{(1,18)}$ = 0.072), (c) caecum lumen (wild-type n = 16, *Nrxn1α* $^{+/-}$ n = 20, p = 0.1265, $F_{(1,28)}$ = 2.480), (d) caecum mucus (wild-type n = 18, *Nrxn1α* $^{+/-}$ *n = 16,* p = 0.7338, $F_{(1,26)}$ = 0.118).

3.2.2.2 Impact of Liraglutide treatment

Liraglutide treatment significantly increases the number of bacterial species in the caecum lumen

Liraglutide treatment significantly increased the number of DGGE bands counted in the caecum lumen layer ($p = 0.0037$, $F_{(1,28)} = 10.034$, Figure 43c), but has no significant effect on the number of bands counted in the caecum mucus layer ($p = 0.0939$, $F_{(1,26)} = 3.024$, Figure 43d).

The number of bands counted in the colon were also similar between saline-treated and liraglutide-treated animals in both the lumen ($p = 0.8077$, $F_{(1,26)} = 0.060$, Figure 43a) and in the mucus layer ($p = 0.3537$, $F_{(1,18)} = 0.907$, Figure 43b) indicating that Liraglutide treatment has no significant effect on the number of bacterial species within the colon.

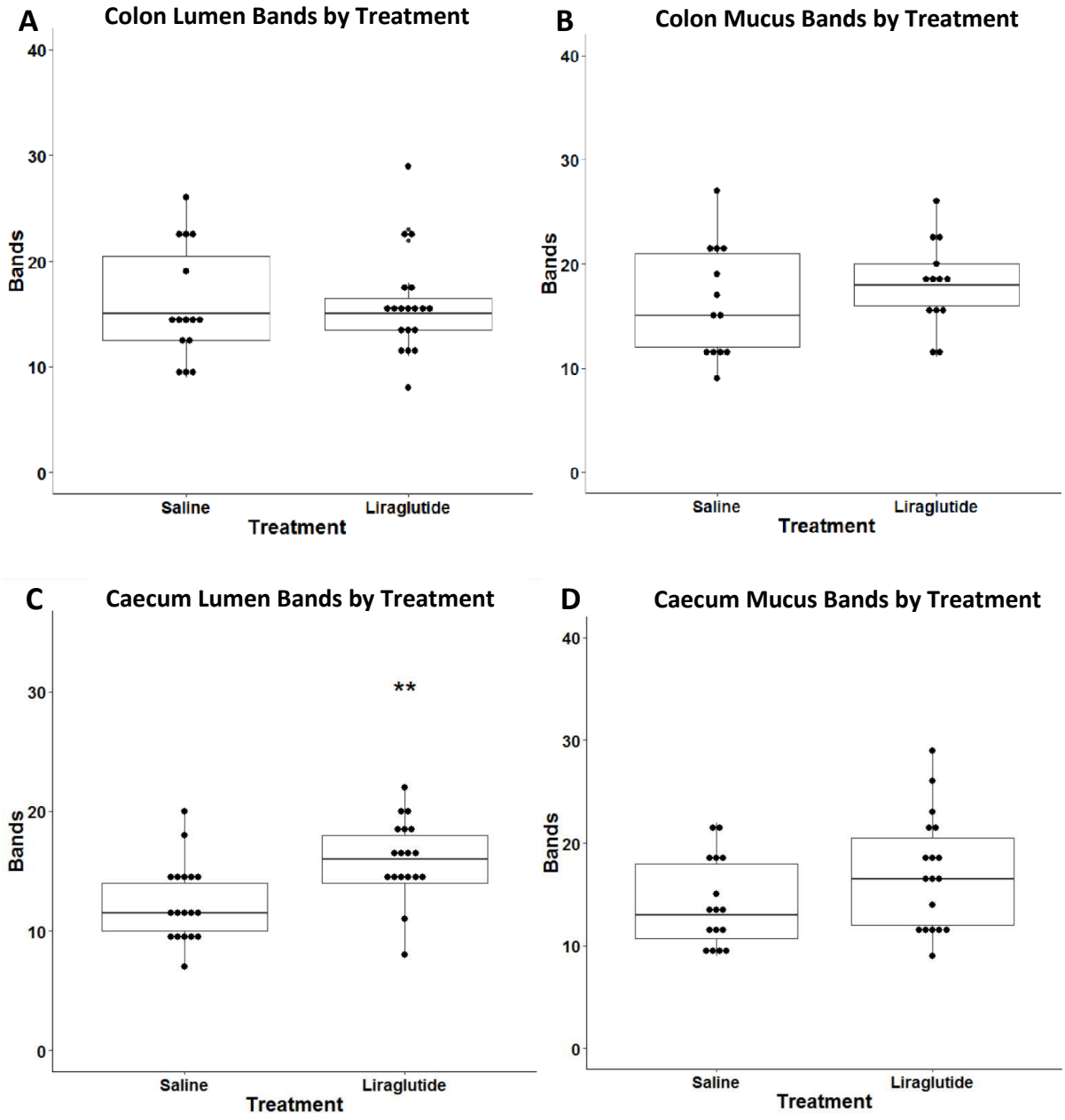


Figure 43: Liraglutide treatment significantly increases the number of bacterial species in the caecum lumen. Data shown as DGGE band counts in the (a) colon lumen (saline n = 15, Liraglutide n = 19, p = 0.8077, F(1,26) = 0.060), (b) colon mucus (saline n = 13, Liraglutide n = 13, p = 0.3537, F(1,18) = 0.907), (c) caecum lumen (saline n = 18, Liraglutide n = 18, p = 0.0037, F(1,28) = 10.034), (d) caecum mucus (saline n = 16, Liraglutide n = 18, p = 0.0939, F(1,26) = 3.024).

3.2.2.3 Impact of Sex

In the colon lumen females had more bacterial species than males

In the colon lumen, females had significantly more bands than males ($p = 0.0331$, $F_{(1,26)} = 5.065$, Figure 44a), although there were no significant differences in the number of bands in the colon mucus layer ($p = 0.0884$, $F_{(1,18)} = 3.245$, Figure 44b). In the caecum, there was a trend for males to have fewer bands than females in the lumen (0.8215, $F_{(1,28)} = 0.052$, Figure 44c), but the number of bands were similar between both sexes in the mucus layer ($p = 0.6898$, $F_{(1,26)} = 0.163$, Figure 44d). Overall, this suggests that females may have a higher number of bacterial species than males. However, this does not replicate what we found in the previous sex independent study (Figure 41a).

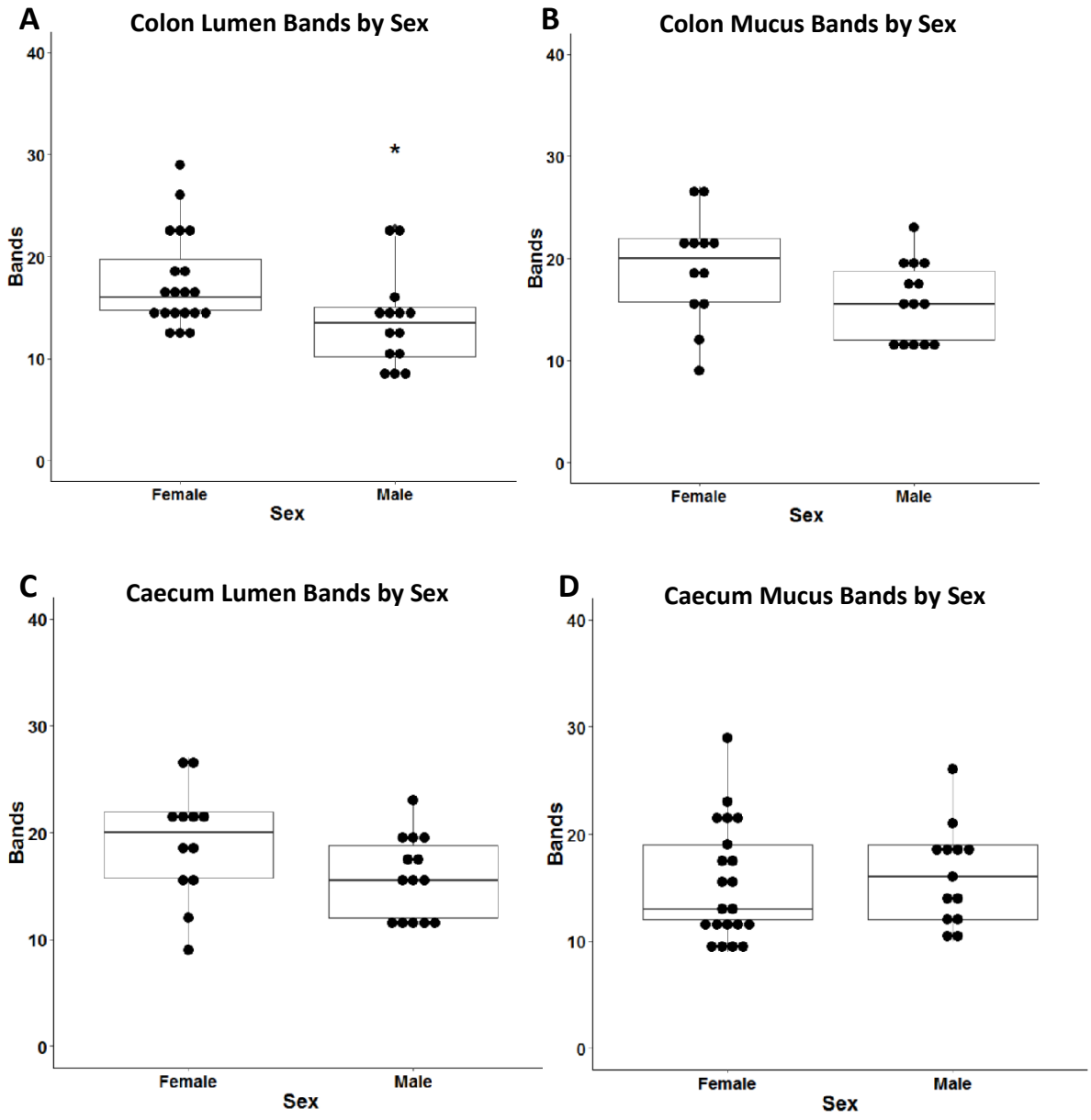


Figure 44: Females had more bacterial species than males in the colon lumen. Data shown as DGGE band counts in the (a) colon lumen (females n = 20, males n = 14, p = 0.0331, F(1,26) = 5.065), (b) colon mucus (females n = 12, males n = 14, p = 0.0884, F(1,18) = 3.245), (c) caecum lumen (females n = 12, males n = 14, p = 0.8215, F(1,28) = 0.052), (d) caecum mucus (females n = 21, males n = 13, p = 0.6898, F(1,26) = 0.163). * P<0.05 compared to relevant control (ANOVA).

3.2.2.4 Genotype, treatment and sex interactions on Bacterial Diversity

*Neurexin1*α$^{+/-}$ genotype does not significantly modify the impact of liraglutide on DGGE band count

DGGE band analysis shows no significant genotype x treatment interactions in the colon lumen (genotype x treatment, $p = 0.852$, $F_{(1,26)} = 0.036$), colon mucus (genotype x treatment, $p = 0.395$, $F_{(1,18)} = 0.759$), caecum lumen (genotype x treatment, $p = 0.551$, $F_{(1,28)} = 0.365$), caecum mucus (genotype x treatment, $p = 0.219$, $F_{(1,26)} = 1.585$).

Sex does not significantly influence the impact of *Neurexin1*α heterozygosity on DGGE band count

There were no genotype x sex interactions on DGGE band count in the colon lumen (genotype x sex, $p = 0.590$, $F_{(1,26)} = 0.298$), colon mucus (genotype x sex, $p = 0.124$, $F_{(1,18)} = 2.661$), caecum lumen (genotype x sex, $p = 0.317$, $F_{(1,28)} = 1.037$), or caecum mucus (genotype x sex, $p = 0.156$, $F_{(1,26)} = 2.138$).

Sex does not significantly influence the impact of Liraglutide on DGGE band count

There were no sex x treatment interactions on DGGE band count in the colon lumen (treatment x sex, $p = 0.653$, $F_{(1,26)} = 0.206$), colon mucus (treatment x sex, $p = 0.590$,$F_{(1,18)} = 0.302$), caecum lumen (treatment x sex, $p = 0.865$, $F_{(1,28)} = 0.030$), or caecum mucus (treatment x sex, $p = 0.783$, $F_{(1,26)} = 0.077$).

3.2.3 Impact of *Neurexin1α* heterozygosity and Liraglutide treatment on the microbiome composition identified through clustering

In our second analysis of the microbiome we used dendrogram clusters and NMDS plots to determine any significant differences in bacterial communities between sample groups, excluding the outliers mentioned previously.

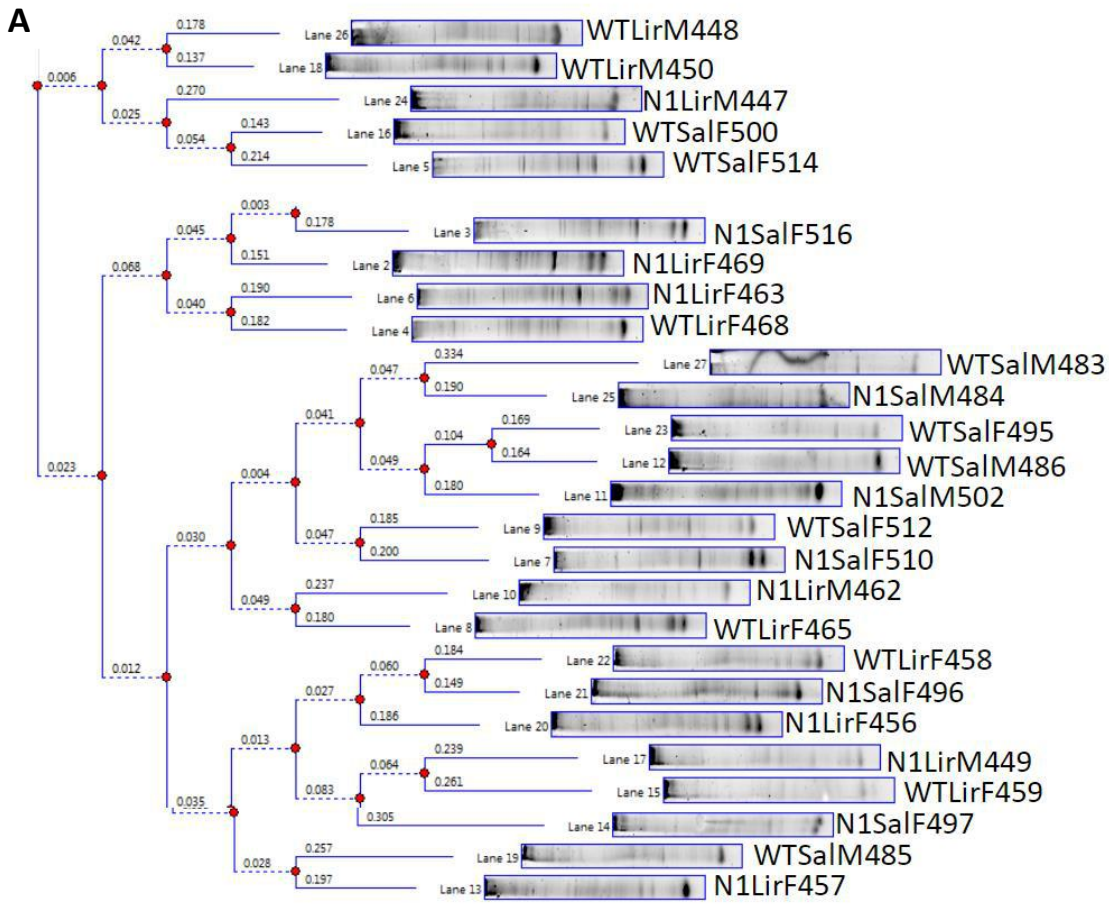
3.2.3.1 *Neurexin1α* heterozygosity significantly changes the bacterial communities in the caecum mucus layer, and in both colon layers

Nrxn1α $^{+/-}$ genotype caused a significant change in microbial clustering compared to wild-type mice in saline-treated animals. This was true in the caecum mucus (Pr(>F) = 0.0626 trend, Figure 46b), the colon lumen (Pr(>F) = 0.03586, Figure 47), and the colon mucus (Pr(>F) = 0.03258, Figure 48b) layers. These suggest that *Nrxn1α* $^{+/-}$ genotype causes a difference in bacterial communities in saline-treated mice. We also saw a significant genotype effect on microbial clustering in Liraglutide treated animals in the caecum mucus (Pr(>F) = 0.04181, Figure 46), the colon lumen (Pr(>F) = 0.03586, Figure 47) layers, which suggests *Nrxn1α* heterozygosity significantly changes the microbiome composition and this is maintained when animals were treated with Liraglutide. However, no significant differences in clustering were observed in the caecum lumen (Figure 45).

3.2.3.2 Liraglutide treatment significantly changes the microbiome composition in the caecum and colon mucus layers

Treatment with Liraglutide significantly altered microbial clustering when compared to saline-treated mice in wild-type animals. These effects were observed in the caecum mucus (Pr(>F) = 0.03423, Figure 46c), and the colon mucus (Pr(>F) = 0.01057, Figure 48) layers, but this was not in the luminal samples from these regions. This suggests that Liraglutide significantly impacts on bacterial composition of the mucus layer, and that this effect is location specific. We also saw an effect of liraglutide treatment in *Nrxn1α* $^{+/-}$ animals where liraglutide induced significant clustering differences in the caecum mucus (Pr(>F) =0.02092, Figure 46), the colon lumen (Pr(>F) = 0.04242, Figure 47b), and the colon mucus (Pr(>F) = 0.04242, Figure 48). No significant effects of Liraglutide treatment were seen in the caecum lumen layer. These suggest that Liraglutide significantly impacts microbial clustering in the colon, and in the caecum mucus layer only. However, as clustering significantly differed between *Nrxn1α* $^{+/-}$-Liraglutide and *Nrxn1α* $^{+/-}$-WT mice, Liraglutide is unlikely to restore the microbiome to a similar composition as wild-type mice.

Caecum Lumen layer



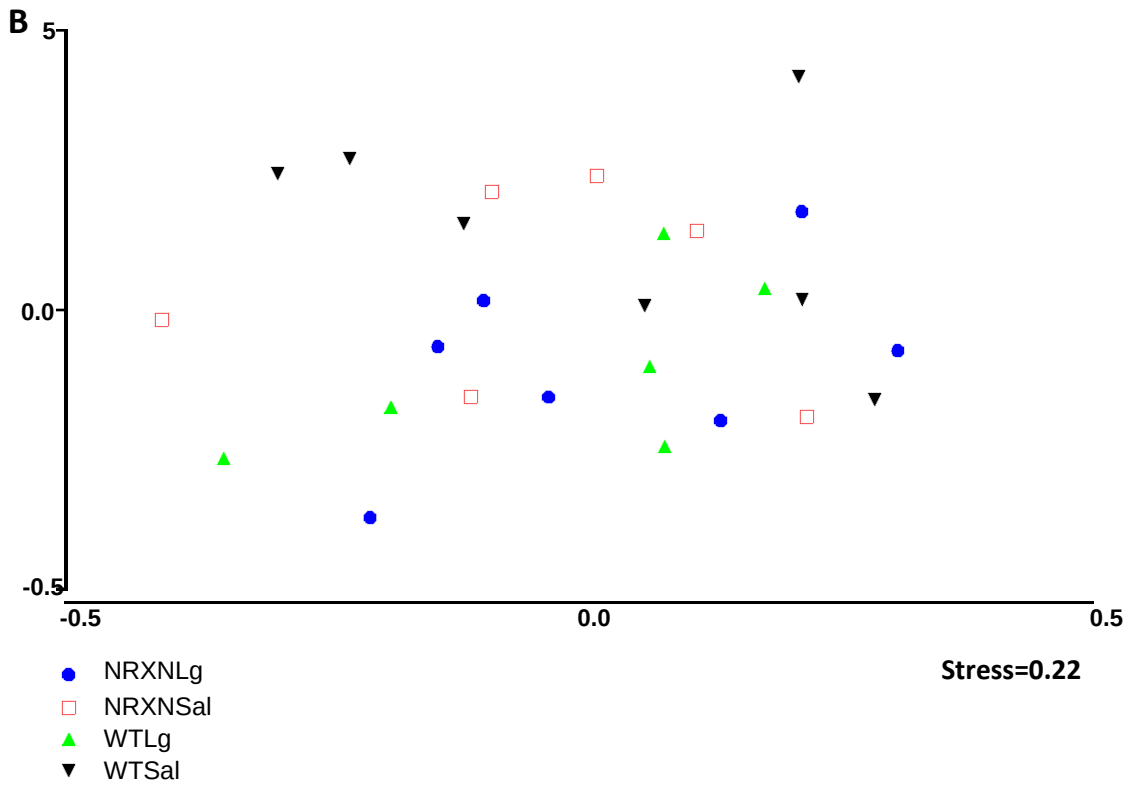
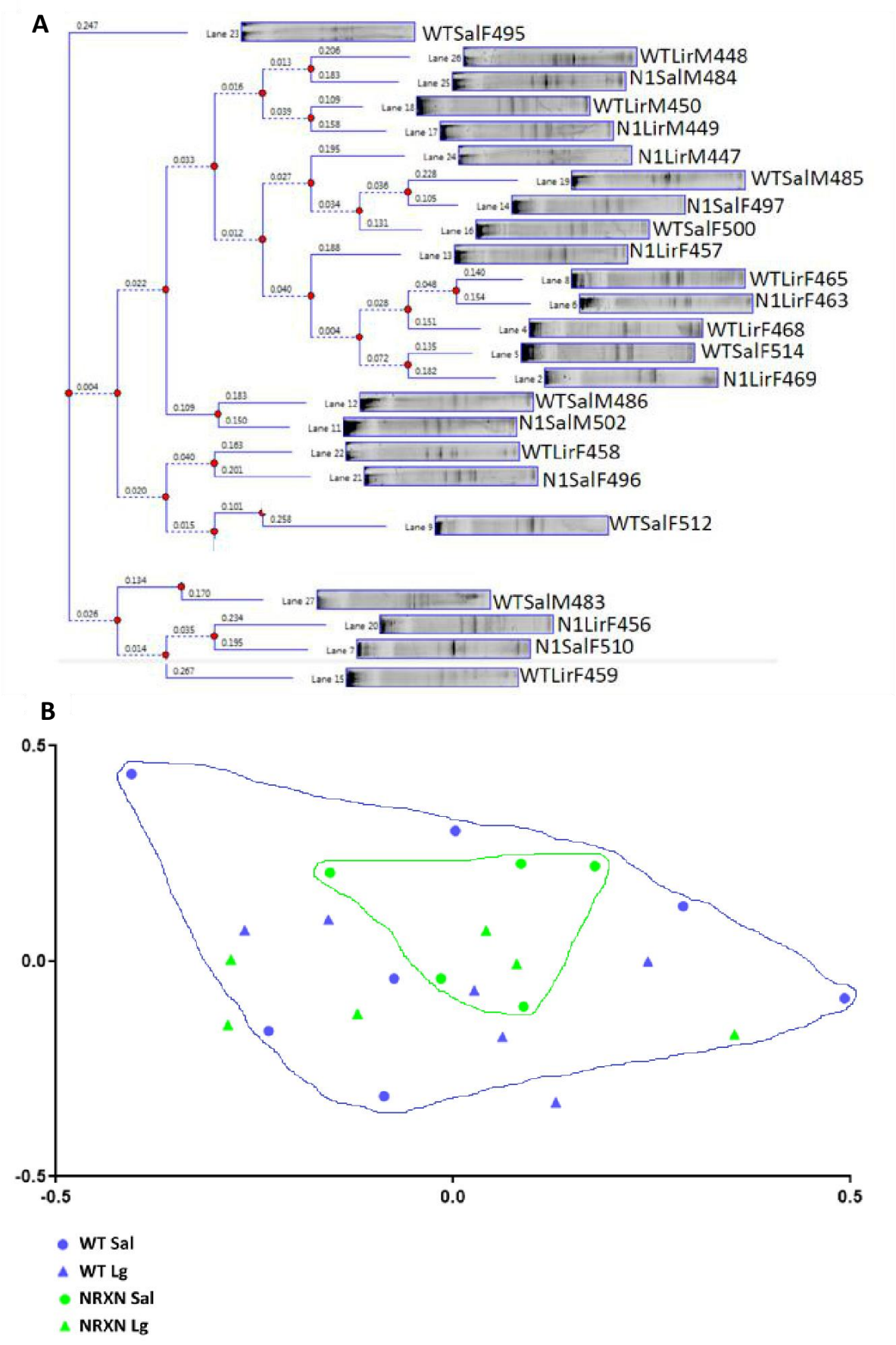


Figure 45: (a) Dendrogram and (b) NMDS plots of DGGE analysis from *Nrxn1α*$^{+/-}$ and wild-type mice, treated with or without Liraglutide from caecum lumen samples. NMDS stress value = 0.22 indicating good quality fit of the data. No significant differences in clustering were observed between the different experimental groups.

Caecum Mucus Layer



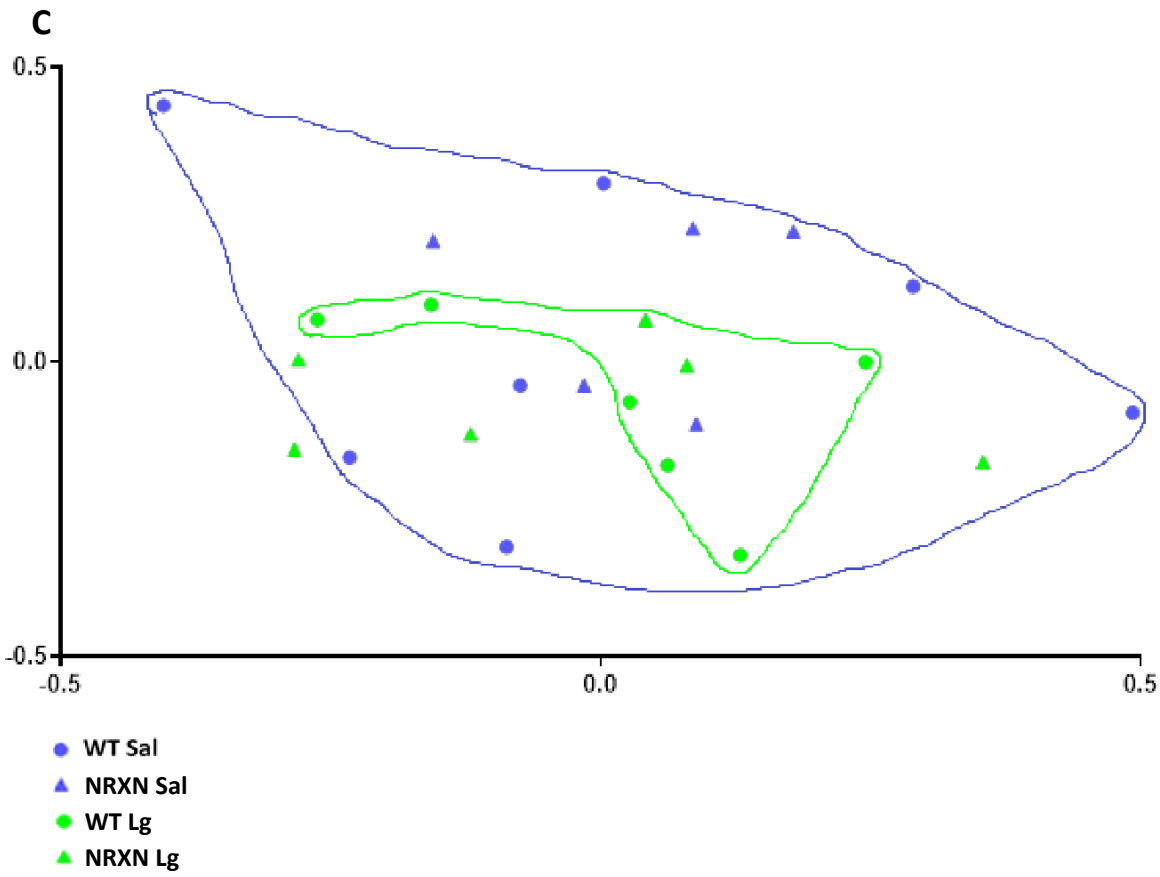
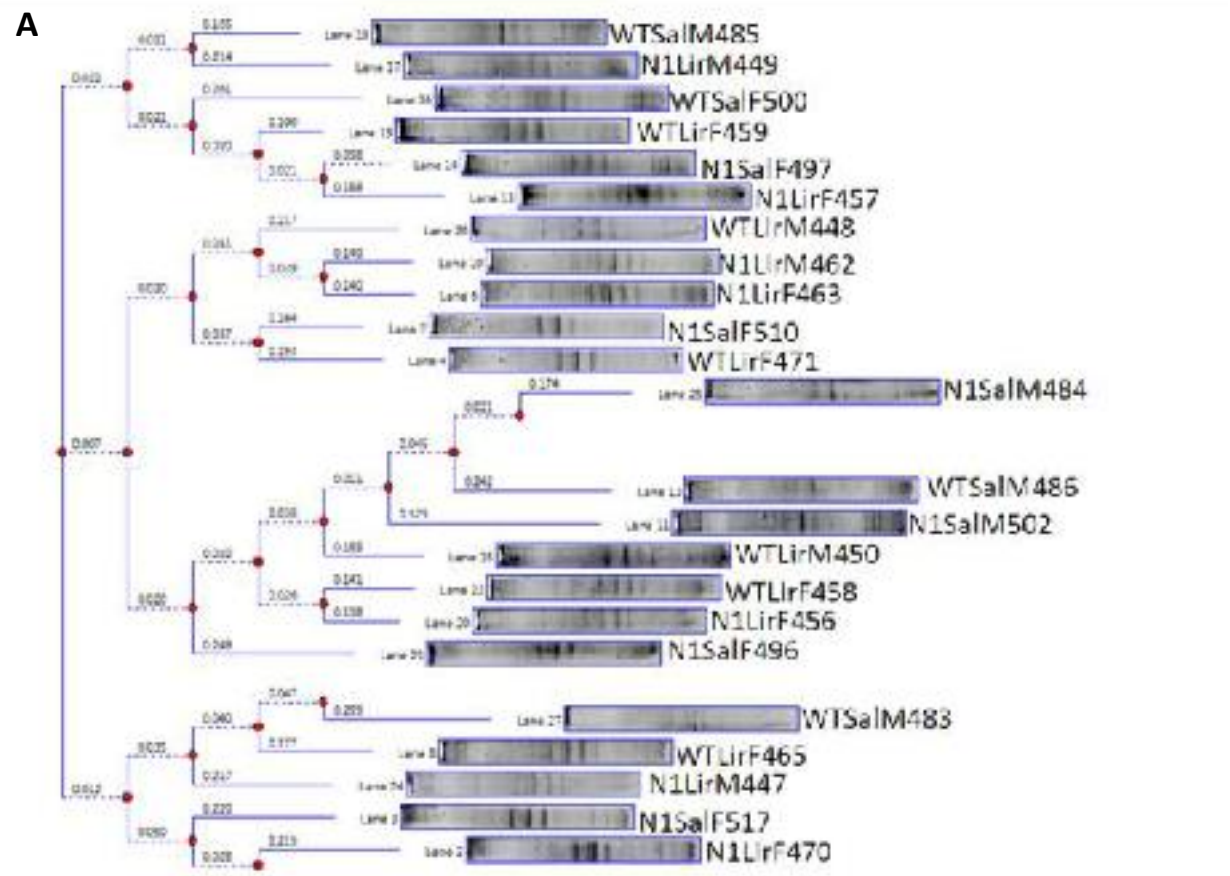


Figure 46: (a) Dendrogram and (b, c) NMDS plots of *Nrxn1α*$^{+/-}$ gene knockouts and wild-type mice, treated with or without Liraglutide, in the caecum mucosal layer. NMDS stress value = 4.42 indicating good quality fit of the data. (b) *Nrxn1α* $^{+/-}$ genotype trend seen between *Nrxn1α* $^{+/-}$-Saline and WT-Saline mice (Pr(>F) = 0.0626), and significant differences observed between *Nrxn1α* $^{+/-}$-Liraglutide and WT-Liraglutide (Pr(>F) = 0.04181). Clustering cloud outline shown for WT-Saline versus *Nrxn1α* $^{+/-}$-Saline shown. (c) Significant effects of Liraglutide treatment were observed between WT-Liraglutide and WT-Saline mice (Pr(>F) = 0.03423), and between *Nrxn1α* $^{+/-}$-Liraglutide and *Nrxn1α* $^{+/-}$-Saline mice (Pr(>F) =0.02092). The clustering cloud outline for WT-Saline versus WT-Liraglutide mice are shown.

Colon Lumen layer



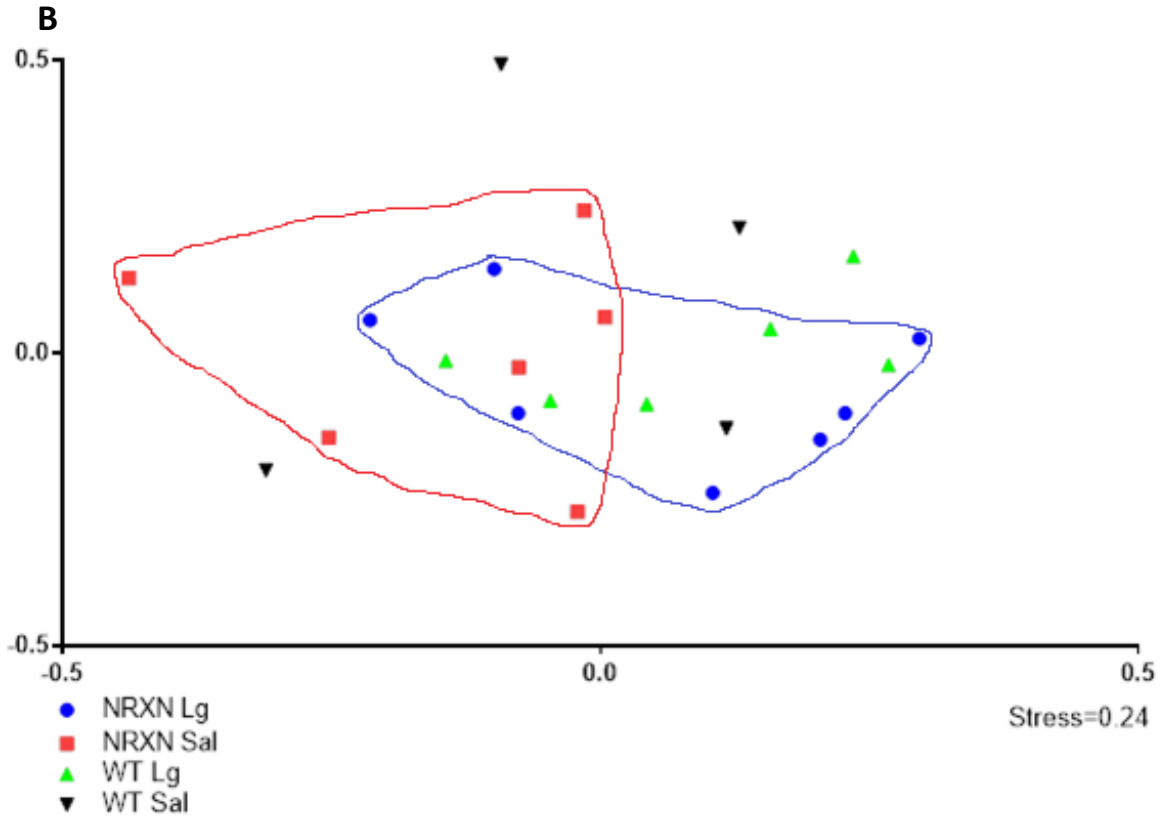
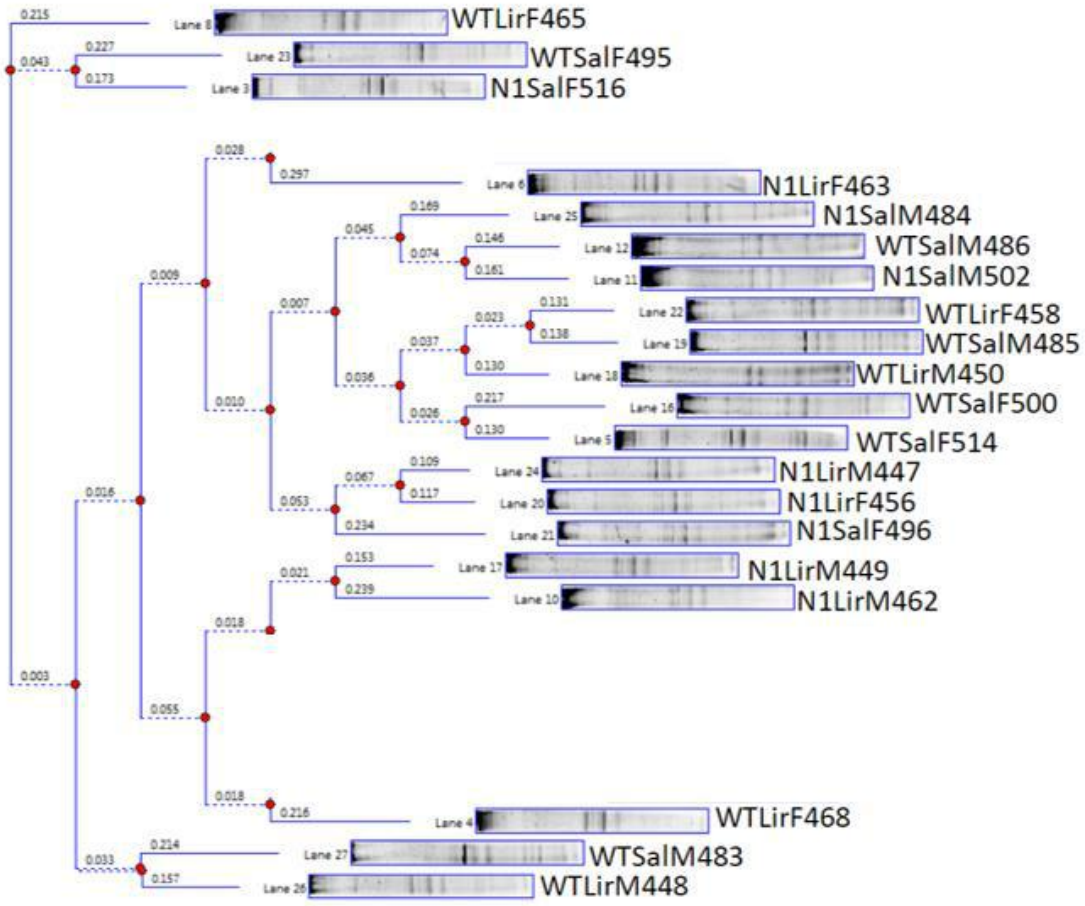


Figure 47: (a) Dendrogram and (b) NMDS plot of *Nrxn1α* $^{+/-}$ gene knockouts and wild-type mice, treated with or without Liraglutide, in the colon luminal layer. NMDS stress value = 4.36 indicating good quality fit of the data. Significant genotype effects observed between *Nrxn1α* $^{+/-}$-Liraglutide and WT-Liraglutide mice (Pr(>F) = 0.03586). A possible trend was observed between *Nrxn1α* $^{+/-}$-Saline and WT-Saline mice (Pr(>F) = 0.0626). Significant Liraglutide effects observed between *Nrxn1α* $^{+/-}$-Liraglutide and *Nrxn1α* $^{+/-}$-Saline mice (Pr(>F) = 0.04242). Significant effects also observed between WT-Liraglutide and WT-Saline mice (Pr(>F) = 0.03423). Clustering cloud shown highlights differences between *Nrxn1α* $^{+/-}$-Saline and *Nrxn1α* $^{+/-}$-Liraglutide treated mice.

Colon Mucus layer



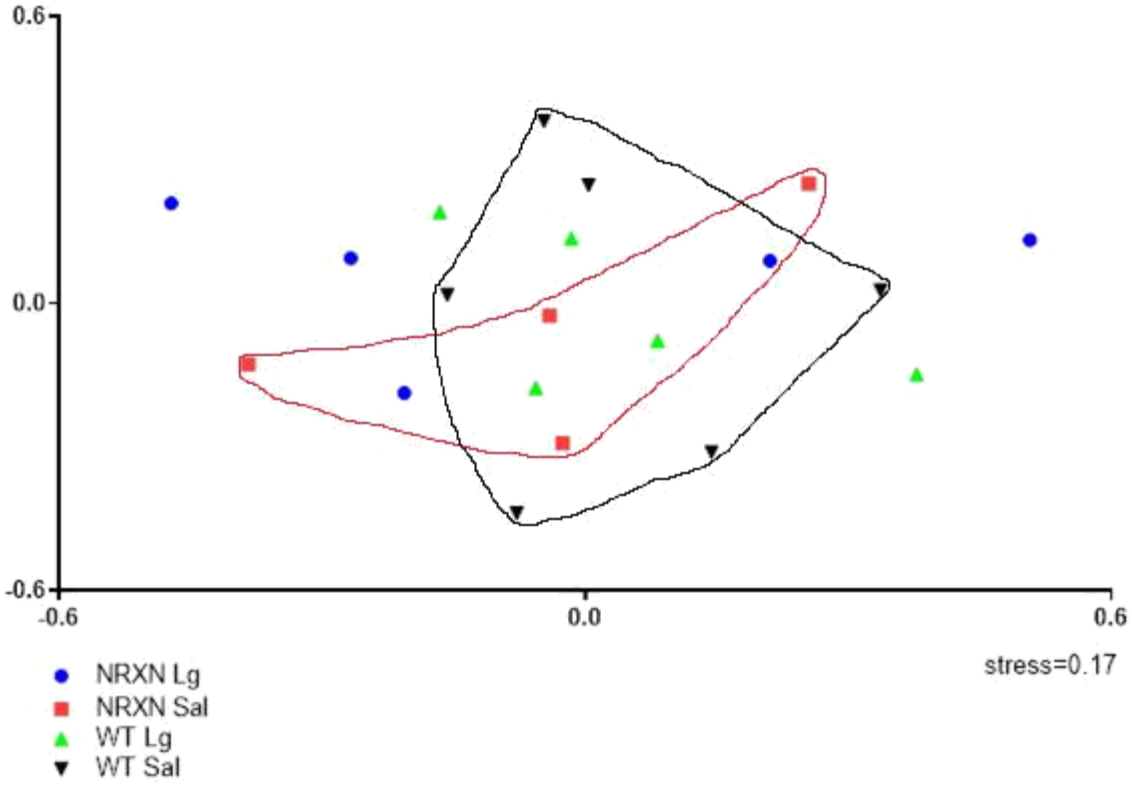


Figure 48: (a) Dendrogram and (b) NMDS plot of *Nrxn1α* $^{+/-}$ and wild-type mice, treated with or without Liraglutide, in the colon mucosal layer. NMDS stress value = 5.27 indicating good quality fit of the data. Significant genotype effects observed between *Nrxn1α* $^{+/-}$-Saline and WT-Saline mice (Pr(>F) = 0.03258). Significant genotype effects were also observed between *Nrxn1α* $^{+/-}$-Liraglutide and WT-Liraglutide mice (Pr(>F) = 0.03586). Significant Liraglutide treatment effects between WT-Liraglutide and WT-Saline mice (Pr(>F) = 0.01057). Significant effects of Liraglutide treatment were also observed between *Nrxn1α* $^{+/-}$-Liraglutide and *Nrxn1α* $^{+/-}$-Saline mice (Pr(>F) = 0.04242). Clustering cloud emphasises the difference between *Nrxn1α* $^{+/-}$--Saline and WT-Saline mice.

3.2.3.4 Summary

Impact of PCP treatment on the microbiome:

There was no evidence that subchronic PCP exposure had a significant impact on the bacterial communities of the gut microbiome as determined from clustering analysis or DGGE band count analysis. However, we found a significant sex effect on the number of bacterial species as indicated by DGGE band number analysis. We found significant evidence for a higher number of bacterial species in the colon lumen of males in comparison to females (Figure 41a). In contrast, we also found significant evidence for a higher number of bacterial species in the colon lumen of females in comparison to males (Figure 44a). These data suggest that there are sex differences in relation to the number of bacterial species in the gut microbiome, although these need to be investigated further to determine sex-specific differences.

Impact of *Neurexin1α+/-* heterozygosity on the gut microbiome:

Nrxn1α heterozygosity had no significant impact on DGGE band count when compared to wild-type mice. However, clustering analysis of bacterial communities in the colon and caecum mucus layer supported an altered microbiome composition in heterozygous *Nrxn1α* mice, suggesting there are differences in bacterial communities. Further investigation will be required to determine more specific effects on the gut microbiome composition and identifying the specific bacteria affected with regards to the effects of *Nrxn1*α heterozygosity.

Impact of Liraglutide treatment on the gut microbiome

Treatment with Liraglutide significantly changed the abundance of bacterial species in the caecum lumen as determined by DGGE band count analysis (Figure 42c). There was no evidence of any significant changes to the number of bands counted in the colon or in the caecum mucus layer. Clustering analysis revealed an altered microbiome composition in Liraglutide-treated mice in the mucosal layers of the colon and caecum, but not in the lumen. We found no evidence that Liraglutide might restore the microbiome composition in *Nrxn1α*$^{+/-}$ mice to a similar state to that seen in wild-type mice as there was a significant difference in cluster analysis between *Nrxn1α* $^{+/-}$-Liraglutide and *Nrxn1α* $^{+/-}$-saline mice.

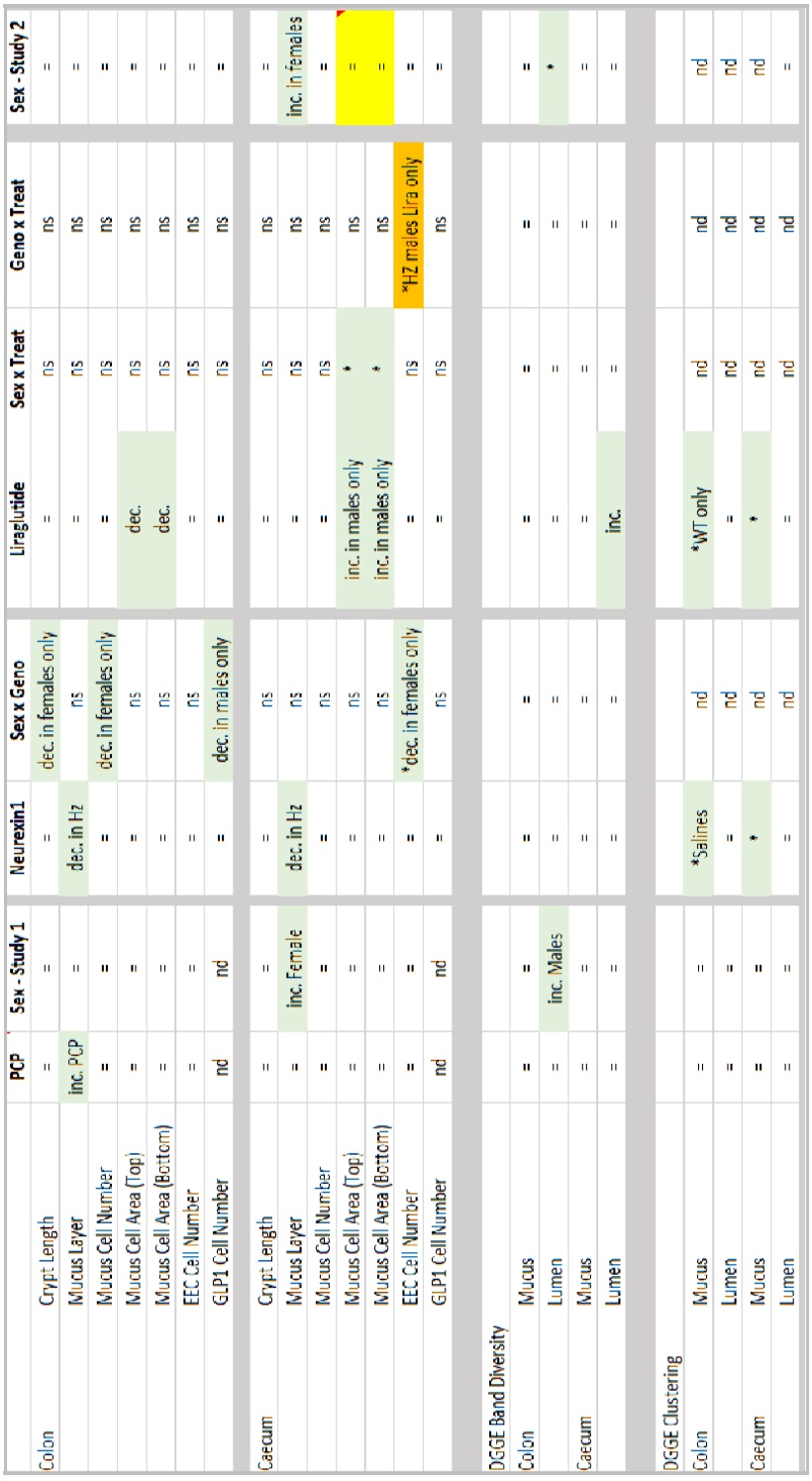
Impact of Sex on the gut microbiome

We concluded that females had significantly more DGGE bands than males in the colon lumen, which suggests females have a higher number of bacterial species present. There was no evidence of sex differences in the colon mucus layer, or caecum lumen or mucus layers. We did not investigate the impact of sex on microbial clustering in this study.

3.3 Summary Table of Results

Table 3: Summary of gut architecture and gut microbiome results. Impact of PCP treatment, *Neurexin1*α heterozygosity, Liraglutide treatment, and Sex on aspects of the gut architecture and microbiome composition in the colon and caecum.

(nd) = not determined; (=) = no effect; (ns) = non-significant; (*) = significant interaction.



		PCP	Sex - Study 1	Neurexin1	Sex x Geno	Liraglutide	Sex x Treat	Geno x Treat	Sex - Study 2
Colon	Crypt Length	=	=	=	dec. in females only	=	ns	ns	=
	Mucus Layer	inc. PCP	=	dec. in Hz	ns	=	ns	ns	=
	Mucus Cell Number	=	=	=	dec. in females only	=	ns	ns	=
	Mucus Cell Area (Top)	=	=	=	ns	dec.	ns	ns	=
	Mucus Cell Area (Bottom)	=	=	=	ns	dec.	ns	ns	=
	EEC Cell Number	=	=	=	ns	=	ns	ns	=
	GLP1 Cell Number	nd	nd	=	dec. in males only	=	ns	ns	=
Caecum	Crypt Length	=	=	=	ns	=	ns	ns	=
	Mucus Layer	=	inc. Female	dec. in Hz	ns	=	ns	ns	inc. in females
	Mucus Cell Number	=	=	=	ns	=	ns	ns	=
	Mucus Cell Area (Top)	=	=	=	ns	inc. in males only	*	ns	=
	Mucus Cell Area (Bottom)	=	=	=	ns	inc. in males only	*	ns	=
	EEC Cell Number	=	=	=	*dec. in females only	=	ns	*Hz males Lira only	=
	GLP1 Cell Number	nd	nd	=	ns	=	ns	ns	=
DGGE Band Diversity									
Colon	Mucus	=	=	=	=	=	=	=	=
	Lumen	=	inc. Males	=	=	=	=	=	*
Caecum	Mucus	=	=	=	=	=	=	=	=
	Lumen	=	=	=	=	inc.	=	=	=
DGGE Clustering									
Colon	Mucus	=	=	*Salines	nd	*WT only	nd	nd	nd
	Lumen	=	=	=	nd	=	nd	nd	nd
Caecum	Mucus	=	=	*	nd	*	nd	nd	nd
	Lumen	=	=	=	nd	=	nd	nd	=

4. Discussion

4.1 Impact of subchronic PCP treatment on the gut

The key finding with regards to subchronic PCP treatment was that we found a significantly thicker muscle layer in the colon of PCP-treated mice (Figure 3a). An increased muscle layer suggests increased peristalsis which is subject to chemical stimuli including neurotransmitters (Spencer et al, 2001), and increased motility can cause changes to the composition of the microbiome (Cremer et al, 2016), however our DGGE cluster analysis and DGGE band analysis showed no significant changes between the experimental groups, hence the mechanism for an increase in muscle layer thickness requires more investigation.

4.1.1 PCP may influence neurotransmitters in the gut to impact on the muscle layer

PCP is an NMDA-R antagonist; these receptors play an important role in microbiota-gut-brain communication. NMDA receptors are found throughout the body on the dendrites of neurons in several areas of the body including in the brain and the gut (Baj et al, 2019). Under normal conditions, neurotransmitters including glutamate bind to NMDA receptors which is important for controlling synaptic plasticity (Nadler, 2012) amongst other functions. It has been proven that NMDA receptors are present in the enteric nervous system (Zhou and Verne, 2008). Studies have shown that alterations to glutaminergic receptor activity along the microbiota-gut-brain axis may influence brain function and behaviour (Baj et al, 2019). Glutamate in the brain is required for sending signals between nerve cells, and under normal conditions it plays an important role in learning and memory (Zhou and Danbolt, 2014). Metabolomic studies have shown that changes in the gut saprophytic microflora are correlated with alterations in glutamate brain levels (Matsumoto et al, 2013). Previous studies show that levels of glutamine, which is involved in the synthesis of glutamate, are lower in the brains of GF mice (Zheng et al, 2019). BDNF, which regulates the expression of NMDA receptors, is also lower in the hippocampus of GF mice as represented by a decreased expression of the GluN2A subunit (Sudo et al, 2004). These suggest that a healthy microbiome is required to regulate glutamatergic transmission in the brain. An altered or absent microbiota can alter glutamatergic neurotransmission in the brain which influences behaviour. PCP has been proven to reduce NMDA receptor binding activity and decrease the expression of NMDA receptor proteins in the brain (Lee et al, 2006). Glutamate is also important for gut motility and secretion (Kirchgessner, 2001), thus

lack of glutamate binding in the brain may compromise intestinal motility. It is possible that a reduction in neuronal induced gut motility could provoke a change to the structure of the muscle layer and hence why PCP-treated mice have a greater muscle layer depth in the colon.

Other neurotransmitters in the brain affected by PCP administration, at least in the CNS, include DA, NE and 5-HT, and ACh (Bey and Patel, 2007). PCP exhibits a high affinity for the high affinity state of the D2 receptors (Kapur and Seeman, 2002). This affinity has been proven to be similar to PCPs affinity for NMDA receptors (Kapur and Seeman, 2002). PCP can inhibit DA reuptake although the mechanisms are not well defined. When reuptake is inhibited, DA cannot be absorbed by the postsynaptic neurone and is blocked from re-entering the presynaptic neuron (Song et al, 2012). This results in increased extracellular concentrations of DA and increase in dopaminergic neurotransmission in the brain which may contribute to its ability to induce psychosis and other schizophrenia-like symptoms (Brisch et al, 2014). Research indicates that PCP mainly exerts it effects through the high affinity D2 receptor (Seeman et al, 2005), and evidence shows that in the absence of this D2 receptor, gut motility is abnormal (Li et al, 2006). The increase in muscle layer depth following PCP administration may suggest a mechanism through which dopamine regulates gut motility.

The neurotransmitter 5-HT is essential for regulating mood, sleep, social behaviour and memory (Jenkins et al, 2016). 5-HT2, a subfamily of 5-HT, have also been shown to modulate the activation of dopaminergic neurons and enhance DA release in the prefrontal cortex (Bortolozzi et al, 2005). PCP increases serotonergic activity in the brain by inhibiting 5-HT uptake (Hori et al, 2000). More than 90% of the body's 5-HT is synthesised in the gut (Yano et al, 2015) where it regulates intestinal movements (Camilleri, 2009). PCP potentially acts to enhance 5-HT levels in the gut which causes an increase in gut motility (Mawe and Hoffman, 2013) in addition to the production and release of gastric and colonic mucus (Ormsbee and Fondacaro, 1985). Studies have shown an acceleration in mucus transport during conditions of excess 5-HT in the gut (Maruyama et al, 1984). These suggest that the increase in serotonergic activity induced by PCP administration may be responsible for an increased peristalsis in the gut which may be linked to an increased depth of muscle layer in the colon of PCP-treated mice.

The re-uptake of NE is also compromised after PCP administration (Bayorh and Kopin, 1982). With regards to the colon, NE normally has an inhibitory effect on the enteric nervous system which decreases gastrointestinal motility (Nezami and Srinivasan, 2010), which would suggest a decrease in muscle layer thickness as there is reduced motility. It is unlikely that PCP impacts on the muscle layer through inhibiting the re-uptake of NE.

Another mechanism through which PCP could induce an increase in muscle layer depth is through binding to Ach receptors in gut. PCP can block the ion channel of the Ach receptor (Albuquerque et al, 1983), potentially inducing a change to the muscle structure surrounding the gut. ACh is the most common neurotransmitter to induce gastrointestinal smooth muscle contractions, thus lack of Ach suggests fewer muscle contractions which indicates a thinner muscle layer depth which contradicts our findings.

Together, excessive concentrations of glutamate, DA, and 5-HT in the brain following PCP administration may compromise the GI system and ultimately cause the thickness of muscle to increase. As we know that PCP interacts with known molecular targets of neurotransmitter systems that are present within the gut, PCP may impact on aspects of gut function by influencing these neurotransmitter systems either directly in the gut or the brain. To investigate these potential mechanisms further, we could investigate the rate of increase in muscle thickness, the effects on gut motility, and changes to the thickness of the muscle layer when mice have elevated levels of the key neurotransmitters following PCP administration. We could also deduce the relevant mechanisms and pathways at the cellular level.

4.1.2 PCP is unlikely to affect the microbiome

There is little previous work with regards to the effects of subchronic PCP on the microbiome. Although, one study suggested an overall increase in *Bacteroidetes* and *Firmicutes* in rats treated with PCP, but the results were not statistically significant (Jørgensen et al, 2015). On the other hand, the same study demonstrated that the defects to locomotor activity induced by changes to the microbiota following PCP administration were restored by ampicillin treatment (Jørgensen et al, 2015). Ampicillin is a hydrophilic antibiotic and penetrates poorly through the BBB (Lutsar et al, 1998), suggesting it has limited direct interaction with the brain. However, it can readily pass into the CSF in the

presence of inflammation (Kramer et al, 1969). These previous results suggest that PCP, in the absence of inflammation, does induce changes to the microbiome, and these changes may contribute to the symptoms and neurophysiology of PNDs, including schizophrenia.

However, in these studies we found no significant changes to the microbiota when subchronic PCP was administrated, as determined by DGGE cluster analysis and DGGE band analysis. This was true for both the colon and the caecum. Thus, the results from this study support previous data which found a non-significant increase in the levels of *Bacteroidetes* and *Firmicutes* (Jørgensen et al, 2015). However, the microbiome of mice treated with PCP should be further investigated to find more supporting evidence. To further characterise the microbiome following PCP treatment, we could identify individual microbial strains from whole-community profiling (Hamady and Knight, 2009) and target them for isolation using amplicon sequencing to identify specific species (Mukherjee et al, 2018). However, our results from this study indicate that PCP exerts its schizophrenia-like symptoms in mice through other mechanisms which are independent of the microbiome.

4.1.3 PCP conclusion

Overall, with the changes to the muscle layer following subchronic PCP treatment in this study, and because PCP induces schizophrenic-like behaviour in mice (Castañé et al, 2015), we can potentially consider the involvement of the muscle layer in the pathophysiology of the disease. We found that subchronic PCP treatment significantly increased the muscle layer depth in the colon, and research indicates this may be caused by the effects of excess neurotransmitters in the brain. Thus, we can conclude that excess neurotransmitters cause structural changes to the gut muscle layer, although with no changes to the microbiome. As our results and previous studies support non-significant changes to the gut microbiota following subchronic PCP treatment, our results indicate that although PCP induces the symptoms of schizophrenia in mice, it is not a representative model of the role of the gut microbiota. We also observed no significant changes to aspects of the gut architecture besides the thickness of the muscle layer. Therefore, PCP exerts its effects through other mechanisms which are unlikely to involve gut dysfunction.

Despite finding no significant changes to the microbiome, the microbiota is still likely to play a role in the physiopathology of schizophrenia. This is because other studies have found a correlation between memory performance and gut microbiota profile (Jørgensen et al, 2015). Rodents with impaired cognitive behaviour correlate with an altered microbiome

function which can be corrected by antibiotic treatment (Frolich et al, 2016). In addition, antipsychotic treatment increases the abundance of *Firmicutes* and decreases *Bacteroidetes* in the gut (Bretler et al, 2019), suggesting some relief of the symptoms may be caused by changes to the gut microbiome. Finally, microorganisms can regulate BDNF and NMDA receptors in the CNS (Maqsood and Stone, 2016), and these receptors are compromised in schizophrenia (Zhu et al, 2017). Therefore, changes to the microbiome are likely to be linked with schizophrenia but this requires more investigation using an alternative model to the PCP mouse model of schizophrenia.

4.1.4 Sex differences in gut architecture and the microbiome

With regards to sex, sex significantly impacted aspects of the gut architecture and microbiome in these studies. The key findings related to sex differences were: in the caecum, females had a significantly thicker muscle layer than males; and in the colon lumen, there was a significantly higher number of bacterial species in males than females. Sex had no significant effect on other parts of the gut architecture including number and size of goblet cells, crypt lengths, or EEC count.

Sex significantly impacted on the microbiome of the colon lumen in this study. Although there were limited differences between DGGE cluster analysis, DGGE band analysis generally revealed more bands in males than females (Figure 41a), which suggests that more bacterial species are present in the colon of males. However, this sex effect was not replicated in our second independent study. It is widely accepted that males and females have differences in their microbiome composition (Haro et al, 2016), and some studies indicate that this is related to the functioning of the immune system; with sex-related differences in immunity proven by studies in GF mice (Fransen et al, 2017). In the absence of an innate immune defence, bacterial groups including *Alistipes, Rikenella*, and *Porphyromonadaceae* are overrepresented in the male microbiota in comparison to that present in female mice (Fransen et al, 2017). Another study which investigated the microbiome in males and females at baseline conditions indicated that 17 bacterial operational taxonomic units (OTU's) were significantly greater in male mice, whereas only 11 OTU's were higher in females (Bridgewater et al, 2017). From these previous findings, we can conclude the microbiota of male mice is generally more diverse than females, which partially supports our findings for a sex dependent effect on the microbiome.

4.2 Impact of *Neurexin1α* heterozygosity on the gut

Nrxnα heterozygous gene deletions increase the risk of developing PNDs. This study is the first direct evidence that *Nrxnα* heterozygosity impacts on the architecture and microbiome of the gut. This proposes a potential mechanism as to how these heterozygous deletions increase the risk of developing schizophrenia and autism amongst other PNDs. Our key findings with regards to the impact of *Nrxn1α* heterozygosity on the gut were: the depth of the muscle layer in *Nrxnα*$^{+/-}$ mice was significantly decreased in both the colon and caecum. In female *Nrxnα*$^{+/-}$ mice only, we observed significantly fewer goblet cells in the colon, fewer EECs in the caecum, and shorter colonic crypts. In contrast, in male *Nrxnα*$^{+/-}$ mice we observed significantly fewer GLP-1 +ve EECs in the colon. With regards to the microbiota, we observed no significant differences in the number of bands counted as determined by DGGE analysis. However, we did observe significant microbiome DGGE clustering differences in *Nrxnα* heterozygous mice.

4.2.1 *Neurexin1α* heterozygosity impacts on the muscle layer and microbiome

We found that *Nrxnα* heterozygous mice had a significantly decreased depth of muscle layer in the colon and caecum as compared to wild-type mice (Figure 16). As gut bacteria are critical for optimal muscle function (Nay et al, 2018), changes in the gut bacterial communities seen in *Nrxnα*$^{+/-}$ mice could also contribute to the thinner muscle layer in these animals. We found that heterozygous *Nrxnα* mice had a significantly different microbial clustering profile compared to wild-type mice (Figure 42, Figure 43, Figure 44), although there were no significant impacts on the overall DGGE band count when compared to wild-type mice (Figure 38). Significant clustering differences were observed between *Nrxnα*$^{+/-}$mice and wild-type mice when they were treated with either saline or Liraglutide, emphasising genotype-dependent effects on the microbiota that were not corrected by Liraglutide treatment. Previous studies have shown that *Nrxnα* heterozygous mice display behavioural deficits relevant to autism or schizophrenia (Dachtler et al, 2015), and other studies evidence an altered microbiota composition in individuals with several PNDs (Reddy and Saier, 2015). Specific behaviours shown by *Nrxnα*$^{+/-}$ mice include a deficit in social memory (Dachtler et al, 2015), a decrease in prepulse inhibition, and an impaired nest-building activity and increased grooming behaviours (Etherton et al, 2009). Manipulation of the microbiome in GF mice also shows behaviours including social deficits

and increased repetitive behaviour (Borre et al, 2014). Furthermore, transferring a microbiota from a schizophrenic patient to GF mice induced similar behaviours to those observed in autistic mouse models. However, thus far there is lacking evidence on the effects of Nrxn$\alpha^{+/-}$heterozygosity directly on the microbiome in humans.

Based on our findings in this study, *Nrxnα* heterozygosity could play a role in inducing schizophrenia and autism-like symptoms by directly altering the composition of the microbiota, although far more evidence is required to determine this possibility. The interaction between *Nrxnα*$^{+/-}$ heterozygosity and microbiota composition is unclear. Previously, there has been a downregulation in the activity of the *Nrxn* gene in the presence of *Bacteroides fragilis* neurotoxic lipopolysaccharides (Zhao et al, 2019), suggesting there is some form of interaction between the *Nrxn* gene and the microbiome, specifically in relation to *Bacteroides* bacteria. In our study, clustering analysis revealed some significant clustering differences in the bacterial populations of *Nrxnα* heterozygous mice. In contrast, we found no significant differences to the number of DGGE bands counted. Previous research indicates that individuals with autism or schizophrenia have fewer bacterial communities (Kelly et al, 2017), although the abundance of certain species can increase significantly. In autism there are fewer *Bifidobacterium* and *Firmicutes* but higher levels of *Lactobacillus, Clostridium, Bacteroidetes, Desulfovibrio, Caloramator* and *Sarcina (Li et al, 2017). Candida* species are also twice as abundant in autism cases than in controls (Strati et al, 2017). High levels of *Clostridium*, which can produce neurotoxins, have been observed in individuals with autism, and these neurotoxins can impact upon the functioning of the CNS (Parracho et al, 2005). In schizophrenia cases, there are higher amounts of *Lactobacillus* and *Bifidobacterium* and *Lactobacillus gasseri* is 400 times more abundant in patients with schizophrenia when compared to controls, as determined from faecal sample analysis (Dickerson et al, 2017). These findings strongly indicate that the bacterial community profile is altered in PNDs. It would prove useful in our study to determine exactly which bacterial species change in abundance and see if this correlates with the previous clinical data. As mentioned earlier, whole-community profiling (Hamady and Knight, 2009) to identify individual strains and targeting them for isolation using amplicon sequencing (Mukherjee et al, 2018) are examples of how we can do this.

4.2.2 The microbiome potentially influences gut barrier dysfunction

As we found *Nrxnα* heterozygosity to influence the microbiome, our findings may indicate some relevance to the changes in behaviour seen in PNDs. The mucus layer which harbours microbes is normally dense and can be compromised by the absence of certain bacterial species such as *Bifidobacterium*, which is required to modulate mucus production by goblet cells (Engevik et al, 2019). A compromised mucus layer may enable bacterial penetration into the circulation (Schroeder, 2019). In the gut, only certain types of bacteria can adhere to the mucus layer and access epithelial cells. These include *Clostridium, Lactobacillus*, and *Bacteroidetes* (Dieterich et al, 2018), and these species have greater influence on the host immune system due to their proximity with host epithelial cells (Dieterich et al, 2018). These particular bacteria have previously been shown to be elevated in autistic and schizophrenic cases (Mohamadkhani, 2018). Therefore, given the observed impact of *Nrxnα* heterozygosity on the gut microbiome it would be useful to determine any effects on these bacterial species in the gut, and their relationship to the microbiome.

The ability of bacteria to penetrate the epithelial barrier is heavily influenced by inflammation (Johansson et al, 2014). Inflammation of the intestinal epithelial layer is corrected with changes to the microbiota (Belizario et al, 2018). Harmful substances produced from these bacteria cause damage to the intestinal epithelial barrier which allows neurotoxic metabolites to enter the circulatory system which initiates an immune response (Zhu et al, 2017). The release of inflammatory cytokines signals to endothelial cells to change the structure of the tight junctions of the BBB, increasing their permeability and initiating alterations to brain structure and function (Pan et al, 2011). Studies show higher levels of inflammatory molecules in schizophrenic individuals. Some of these include IL1β, TNFα, IL-6 and IL-8 (Pan et al, 2011). Therefore, the change in microbiome composition we observed in *Nrxnα*$^{+/-}$ mice may be related to schizophrenia through decreasing the thickness of the mucus layer followed by bacterial translocation (influenced by inflammation) and interactions with the brain. An area of future work is to investigate the impact of *Nrxnα* heterozygosity on the thickness of the mucus layer.

The absence of certain bacterial species can also compromise the production of SCFAs, which are bacterial metabolites required for the expression of MUC2 (Burger-van Paassen et al, 2009), the release and modulation of mucosal neurotransmitters, and influence on

mucosal immune function (Carabotti et al, 2015). SCFAs supplementation in mice has been shown to ameliorate the psychosocial stress which increases intestinal permeability and induces decreased anxiety-like behaviours as shown in open field tests and forced swim tests (Van de Wouw et al, 2018). These observed behaviours are similar to those seen in *Nrxnα* heterozygous mice. SCFA supplementation also decreases gene expression of the receptors involved in stress signalling in the hypothalamus, hippocampus and colon (Van de Wouw wt al, 2018). Overall, our study supports the likely influence of *Nrxnα* heterozygosity on the microbiome, but further research is required to determine which species are specifically affected. Our data also suggests that the *Nrxnα* heterozygous mouse model is a reliable model for determining the involvement of gut dysfunction with regards to PNDs.

4.2.3 Impact of sex on the gut architecture and the microbiome

We found a significant reduction in goblet cell number in the colon of female *Nrxnα* heterozygous mice which correlates with the declined depth of the muscle layer. However, this effect was not observed in male *Nrxnα* heterozygous mice. It could be presumed that female mice were more sensitive to the gene knockout, however other studies have proven *Nrxnα* induced phenotypes to be more pronounced in male mice (Laarakker et al, 2012), for example increased aggression (Grayton et al, 2013). This agrees with the increased prevalence and severity of symptoms of schizophrenia (Iacono and Beiser, 1992) and autism (Loomes et al, 2017) as seen in males. However, we also observed a significant reduction in EEC number and crypt length which was sex-specific to heterozygous females. One study did show that *Nrxnα*$^{+/-}$ knockouts exhibited significantly reduced locomotor activity and significantly smaller nest building weights in females compared to males (Grayton et al, 2013), which suggests certain phenotypic traits are more pronounced in a sex-dependant manner. This is supported by a study in a mouse model of autistic mice, which showed significant differences in dysbiosis between the following genera: *Bacteroides, Parabacteroides, Sutterella, Dehalobacterium* and *Oscillospir* (Coretti et al, 2017). These genera were found to be key influencers of sex-dependent behaviour regarding behaviour, increased gut permeability and colon proinflammatory state.

4.2.3.1 Sex impacts on EEC number

Additionally, another sex-dependent genotype effect was observed regarding the total number of EECs counted in the caecum, which was significantly reduced in females only.

EECs communicate with nerve cells through hormone secretion (Ye and Liddle, 2017), and neuropods provide a direct connection between EECs and neurons which line the colon (Bohórquez et al, 2015). Electron microscopy has also revealed small clear synaptic vesicles suggestive of organelles for neurotransmission (Bohorques et al, 2015). Evidence suggests that hormones diffuse throughout the lamina propria until they reach the bloodstream or act on vagal afferent nerves (Bohórquez et al, 2015). EEC synapses contact vagal neurons by using glutamate as a neurotransmitter (Kaelberer el al, 2018). The vagus nerve controls the parasympathetic nervous system (PNS) and brings afferent information from organs which include the gut and send signals to the brain. As the enteroendocrine system participates in the microbiota-gut-brain axis, any changes to the microbiota will impact upon the signals in which the brain receives. Microbial-derived molecules including SCFAs and tryptophan metabolites can propagate signals through interacting with EECs (Martin et al, 2018), although some cross the intestinal barrier, enter circulation and may cross the blood-brain-barrier (BBB) as was discussed previously. However, it remains poorly understood whether these molecules reach the brain, or whether they exert effects through vagal afferent signalling. In this study we found fewer EECs in the caecum of *Nrxnα*$^{+/-}$ female mice. Perhaps females were more sensitive to the *Nrxnα*$^{+/-}$gene knockout, specially leading to fewer EEC numbers in the caecum, which seems likely given our other observations on the gut architecture. We saw no significant differences in EEC number in *Nrxnα*$^{+/-}$ male colon or caecum, nor *Nrxnα*$^{+/-}$female colon. Fewer EECs may indicate impaired enteroendocrine signalling to the brain via the vagus nerve (Dockray, 2013), which may impact upon the behaviour we observe in schizophrenia (Rogers et al, 2016). Electrical vagus nerve stimulation could potentially be used as a treatment for schizophrenia (Ardesch et al, 2007). And as enteroendocrine signalling is influenced by the microbiota, the clustering differences we observed in the heterozygous *Nrxnα* animals may also result in impaired vagal signalling, potentially reaching the brain or other parts of the body, likely contributing to the pathophysiology of PNDs.

The levels of the enteroendocrine secretory product, GLP-1, was also investigated in this study. Despite fewer EECs in females, we surprisingly found significantly less GLP-1 +ve cells in the colon of *Nrxnα* heterozygous males but not females. This suggests that GLP-1 levels released by these cells may be reduced in *Nrxnα* heterozygous mice. GLP-1 agonists such as Liraglutide have been targeted for managing the symptoms of schizophrenia, which will be

discussed later (see discussion section 4.3). It remains unclear why there was a sex-dependant genotype effect of less GLP-1 secretion in this study, but there have been links between the gut microbiota influencing GLP-1 secretion. For example, certain bacteria including *Bifidobacterium, Lactobacillus, Lactobacillus reuteri*, and *Akkermansia muciniphila,* contribute to the modulation of GLP-1 (Cornejo-Pareja et al, 2019). From our clustering analysis, we found a significant genotype effect of *Nrxnα* heterozygosity on microbiome clustering, but we were not able to determine any sex-specific effects in this analysis. Despite this, our DGGE band analysis revealed no significant genotype differences but we found males to have significantly fewer bands than females in the colon lumen overall, suggesting there are fewer bacterial species in males than females. The decrease in microbiome clustering, potentially induced by $Nrxn\alpha^{+/-}$, in males could explain the significant reduction in the GLP-1 +ve EECs we observed.

Overall, our findings suggest that aspects of gut dysfunction induced by *Nrxnα* heterozygosity are significantly influenced by sex, with the effect in females generally being more pronounced than in males. Although the depth of the muscle layer and the gut microbiome were not significantly influenced by sex, they were significantly altered in $Nrxn\alpha^{+/-}$ animals as compared to wild-type controls, suggesting *Nrxnα* heterozygosity impacts on many aspects of gut function. These findings may provide a pathway through which deletion of *Nrxn1* increases the risk of developing PNDs, but still requires further investigation.

4.3 Impact of Liraglutide Treatment on the Gut

Liraglutide treatment had a significant effect on the composition of the microbiome, however it did not reverse the impact of *Nrxn1α* heterozygosity to a similar state as that seen in wild-type mice. Liraglutide treatment also significantly decreased the area of goblet cells within the top and bottom layer of colon crypts in animals of both sexes. By contrast, liraglutide caused an increase in goblet cell size in the caecum, although this was seen selectively in males and not seen in females; this was perhaps due to a greater sensitivity for liraglutide in males and thus more pronounced effects. The ability of liraglutide to alter goblet cell size may indicate that the secretion properties of the goblet cells are modified by liraglutide. For example, a decrease in goblet cell size is potentially suggestive of increased mucus secretion from these cells. It is uncertain why Liraglutide had opposing effects on goblet cell size in the colon and caecum, but this suggests the effects of Liraglutide on goblet cell size is location specific.

4.3.1 Liraglutide impacts on the number of EECs

We also investigated the abundance of EECs and GLP-1 positive EECs in the colon and caecum of wild-type and *Nrxn1α*$^{+/-}$ mice. We found that the total number of EECs was significantly decreased in the caecum of *Nrxn1α*$^{+/-}$ male but not female mice, which suggests Liraglutide treatment on EECs in the caecum is both genotype and sex specific. However, despite Liraglutide being a GLP-1 agonist, which might be expected to influence GLP-1 cell number in the gut, we found that Liraglutide treatment had no significant effect on GLP-1 positive EEC number in either the colon or caecum.

4.3.2 Liraglutide is unlikely to impact on goblet cells

There has been no previous research on the effects of Liraglutide treatment on the size of goblet cells in the gut. However, a study by Viby et al, 2013 found goblet cell hyperplasia in cells situated along the epithelium of the airways in mice treated with Liraglutide, which parallels the impact of liraglutide treatment on goblet cell size in the caecum of male mice in this study.

We also found no changes to the number of goblet cells following Liraglutide treatment. In contrast to our findings, previous studies have shown an increase in the number of goblet cells when obese mice were treated with liraglutide (Moreira et al, 2018) which was

correlated with a thicker mucus layer, which is likely to harbour more bacteria (Sicard et al, 2017). However, in our study there was no change in goblet cell abundance, suggesting that there may be some interaction between obesity and the impact of the liraglutide on gut function. This certainly warrants further investigation.

4.3.3 Liraglutide impacts on the microbiome composition

We found a signficiant influence of Liraglutide on gut microbiome composition, as evidenced by altered bacterial population clustering analysis and band count analysis in the DGGE experiments. In the mucus layers of the colon and caecum, we found signficant differences in microbial clustering between saline and liraglutide treated animals, which indicates the presence of different bacterial communities. The microbial populations in the mucus layer remain fairly consistent across time, in contrast to those present in luminal samples (Li et al, 2015). However, we also found a signficantly higher number of DGGE bands in the lumen of the caecum lumen, supporting altered microbial composition in response to liraglutide treatment here too. Overall, our findings support a role of Liraglutide in modulating the composition of the gut microbiome. This is supported by other studies which have shown microbiome alterations following Liraglutide treatment. For example, previous work has shown Liraglutide to modulate gut bacteria through increasing the abundance of *Proteobacteria* phylum, and by increasing the ratio of *Firmicutes* to *Bacteroidetes* (Wang et al, 2016). Decreases in the genera *Roseburia, Erysipelotrichaceae Incertae Sedis, Marvinbryantia*, and *Parabacteroides* have also been observed following treatment with Liraglutide (Wang et al, 2016). Furthermore, they found that lean-related microbiome genera such as *Blautia* and *Coprococcus* were enriched following liraglutide treatment (Wang et al, 2016). However, these microbiota changes were not reproduced by other GLP-1 agonists such as Saxagliptin (Wang et al, 2016). The techniques used in our study did were not able to reveal the specific genera of *Bacteroidetes* and *Firmicutes* which were affected by liraglutide administration, but this is an area for future investigation. 16S ribosomal RNA (rRNA), which codes for the 30S subunit of the bacterial ribosome, sequencing can be used for bacterial detection and identification and can be used to differentiate between closely related bacterial species (Kim et al, 2014). It would be interesting to see if the bacterial changes found in our study correlate with previous work, along with investigating the dose-dependent effects of Liraglutide.

4.3.4 A role for the gut in Liraglutides treatment of Diabetes

As Liraglutide is a GLP-1 analogue drug used in the treatment of type II diabetes, our data suggest that it is possible it exerts its effects, in part, through changes to gut function and the microbiome. As GLP-1 induces the incretin effect (MacDonald et al, 2002), studies have investigated the effects on the gut microbiota under enhanced incretin action. In addition to the changes in *Bacteroidetes* and *Firmicutes* as mentioned previously, studies also show that Liraglutide treated mice have enriched SCFA producing bacteria such as of *Bacteroides acidifaciens* and *Lachnoclostridium* in the gut (Zhang et al, 2018). These SCFAs potentially may act to enhance the secretion of PYY and GLP-1 (Covasa et al, 2019), increase insulin sensitivity in the body (Canfora et al, 2015), and prevent low-grade inflammatory responses (Puddu et al, 2014). Inflammation is a cause of protein misfolding and endoplasmic reticulum stress inside the cell which upregulates the unfolded protein response which can shut down mucin biosynthesis (McGuckin et al, 2010), which may induce a shift in bacterial communities. Hence Liraglutide treatment may change the microbiome composition required for healthy metabolic function. Studies generally show a decrease in bacterial communities following Liraglutide treatment (Zhao et al, 2018) which contrasts with our findings from our DGGE band analysis. Another study showed that Liraglutide treatment increased the levels of active GLP-1 in humans and this delayed the rate of gastric empyting and reduced gut transmit time, potentially affecting the local pH and nutreint composition of the luminal environment of the colon (Zhou et al, 2018). These factors may contribute to the altered microbiome composition. Therefore, it is possible that Liraglutide treats type II diabetes through manipulating the microbiome, yet future work is required to prove this.

With regards to PNDs, Liraglutide may directly interact with neurons within the arcuate nucleus or other sites in the hypothalamus due to its ability to cross the BBB and enhance the regulation of glucose metabolism (Bae and Song, 2017) which potentially improve cognitive dysfunction. However, it is more likely that Liraglutide acts on afferent neurons which innervate the GI tract which signal to the caudal brainstem or enteric neurons (Hayes et al, 2011) which regulates metabolic disorders. Due to the high comorbidity rate between metabolic disorders and schizophrenia (Henderson, 2005), using Liraglutide as a treatment to improve metabolic symptoms may improve cognitive function at the same time.

5. Final Conclusions

The results show that subchronic PCP administration had no significant effect on the gut architecture or microbiome, suggesting that the model does not reproduce the gut dysfunction observed in schizophrenia. Therefore, the schizophrenia-like symptoms induced by PCP are likely to be independent of effects on gut function and microbiome composition.

Nrxn1α heterozygous deletions increase the risk of developing schizophrenia and autism and this study is the first evidence that this genetic alteration impacts on the gut. These effects warrant further investigation in order to ascertain the impact of this deletion.

Liraglutide treatment significantly affected the gut architecture and microbiome composition but did not reverse the impact of Nrxn1α heterozygosity, despite our previous observations that the drug can improve cognitive function in Nrxn1α heterozygous mice. However, the effects of Liraglutide on the gut and microbiome may be relevant to its effects in the treatment of type II diabetes.

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