MICROBIOTA-GUT-BRAIN SIGNALLING

ALTERATIONS IN THE INTESTINAL MICROBIOTA CAN BE DETECTED BY AND INFLUENCE SPECIFIC BRAIN REGIONS

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ABSTRACT

Emerging evidence indicates that the commensal microbiota communicates with the brain and influences behavior. In animal models, perturbation of the microbiota is accompanied by changes in brain-derived neurotrophic factor (BDNF) levels in the brain. However, underlying mechanisms are unknown. We investigated whether vagal-parasympathetic and sympathetic branches of the autonomic nervous system are involved in the microbiota-gut-brain signalling and attempt to identify specific brain regions that are responsive to alterations in the intestinal microbiota. Specific pathogen-free Balb/c mice, with or without surgical vagotomy or chemical sympathectomy, received oral non-absorbable antimicrobials (ATM) *ad libitum* for 7 days. Behavior was tested on day 7 in the light/dark preference and step-down latency tests. Specific brain regions were sectioned and stained for the neuronal activation marker, *c-fos*. Perturbation of the microbiota significantly enhanced the exploratory behavior of mice in both tests and increased the expression of *c-fos* and phosphorylated *c-fos* in the hippocampus and dentate gyrus. *c-fos* expression in the nucleus of the solitary tract was unaffected and neither vagal-parasympathetic nor sympathetic neurotransmission were required for induction of the behavioral change following perturbation of the microbiota. Instability of the commensal microbiota enhances the activation of the hippocampal formation and influences host behavior in a manner that is independent of vagal-parasympathetic and sympathetic autonomic neurotransmission.

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- JC

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ANS	Autonomic Nervous System
ATM	Antimicrobial
BDZ	Benzodiazepine
BL	*Bifidobacterium longum*
BDNF	Brain-Derived Neurotrophic Factor
CCHS	Canadian Community Health Survey
CNS	Central Nervous System
DG	Dentate Gyrus
DRG	Dorsal Root Ganglia
ENS	Enteric Nervous System
GABA	γ-aminobutyric acid
GBA	Gut-Brain Axis
GI	Gastrointestinal
HPA	Hypothalamic-pituitary Adrenal
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IP	Intraperitoneal/Intraperitoneally
MBSC	Mouse Brain Stereotaxic Coordinates
PBS	Phosphate-Buffered Saline

PNS	Peripheral Nervous System
PSNS	Parasympathetic Nervous System
NE	Norepinephrine
NTS	Nucleus of the Solitary Tract
ROI	Region of Interest
SNS	Sympathetic Nervous System
SPF	Specific Pathogen-Free
TH	Tyrosine Hydroxylase
TNBS	2,4,6-trinitrobenzenesulfonic acid
UC	Ulcerative Colitis

INTRODUCTION

The Intestinal Microbiota

The mammalian gastrointestinal (GI) tract is colonized by a multitude of commensal and opportunistic microbes collectively referred to as the intestinal microbiota. Colonization is initially achieved during the process of birth and shortly after the process begins, the host is thought to harbour up to 10^{12} microorganisms per gram of intestinal contents in the densely colonized distal regions of the GI tract[1]. Exposure to such a bacterial load initiates a number of physiological adaptations that persist throughout life and maintain a significant, yet beneficial influence on the host. These include the proper maturation of the GI mucosal immune system, enhancing nutrient acquisition, strengthening the integrity of the intestinal barrier and providing defence against pathogenic microorganism[2]. The intestinal microbiota is becoming more commonly referred to as a metabolically active organ[3] and it is well established that there exists a dynamic relationship between the host and its resident microbiota, particularly in relation to the health of the host. The abundance of genes contained within this microbial organ far outweighs the genetic content of the host, by an estimated factor of 1-2[4] and it is conceivable that for these reasons the microbiota is able to maintain such a powerful influence on the host, particularly on GI and GI-related functions.

Microbiota-Host Interactions

There has been a recent emphasis on the extraintestinal influences of the microbiota, in particular on the central nervous system (CNS). Early evidence demonstrated that challenging animals orally with a gram-negative pathogen *Campylobacter jejuni* results in the onset of anxiety-like behavior[5], suggesting that certain pathogenic bacteria introduced into the gut environment were capable of influencing host behavior. It is also worth noting that a similar relationship between exposure to a pathogen and its ability to influence host behavior was observed in the context of *Citrobacter rodentium* challenge[6]. Functional activation mapping using the transcription factor *c-fos* revealed that a number of brain nuclei were acutely activated (6-7 hours post-challenge) in response to *C. jejuni* in the gut, including the amygdala, paraventricular hypothalamic nucleus, and a number of nuclei within the brainstem[7]. These findings highlight a role for pathogenic bacteria in gut-brain signalling, but the questions remains as to whether or not the collective intestinal microbiota is capable of influencing the CNS. Improvements in the housing and manipulation of experimental animals have profoundly impacted our capacity to study the effects of the intestinal microbiota. In germ-free mice, which lack an endogenous microbiota, it has been demonstrated that a number of genes and related proteins involved in synaptogenesis and long-term potentiation in the brain are differentially expressed in comparison to colonized mice[8]. Additional studies have revealed functional differences of the hypothalamic-pituitary-adrenal (HPA)

axis and its programmed response to stressful stimuli depending on the presence or absence of the microbiota[9]. In comparison to colonized mice, germ-free mice exhibit an exaggerated HPA axis response to restraint stress, which can be reversed by colonization with the probiotic *Bifidobacterium infantis*[9], highlighting that even probiotic bacteria maintain the ability to influence CNS function, albeit through an unidentified mechanism. However, the vast majority of work in this area has involved either pathogenic or probiotic bacteria. The influence of the commensal microbiota on CNS function has remained a relatively untouched area of research and may hold clues to the pathogenesis of a number of microbiota-related disorders, particularly chronic bowel disorders and the associated psychiatric co-morbidities.

IBD and IBS

Chronic bowel disorders, such as Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS), are two important clinical entities thought to include a strong microbial-related etiologic component. IBD is described as a relapsing and remitting condition that involves symptomatic, chronic inflammation along various portions of the GI tract[10]. IBS is a condition characterized by abdominal pain or discomfort that is accompanied by at least two of the following: relief by defecation, change in frequency of stool, or change in consistency of stool[10]. Although well characterized from a symptomatic point of view, the etiology of both conditions is poorly understood. However, it is becoming clear that the intestinal microbiota may play a strong role in their etiology. With more recent advances in

molecular biology, it has been demonstrated that patients suffering from IBS exhibit temporal instability of the intestinal microbiota, characterized by different microbial profiles from analyzed samples of the same individual on different occasions[11]. This idea of microbiota instability is reflected from studies highlighting shifts in species variation[12] and the relative proportion of species present[13]. However, the question remains as to whether or not the observed instability of the microbiota is a driving factor in the clinical manifestation of the disease, or whether it is a mere consequence of the underlying immune activation and subsequent inflammation.

It is well documented that IBD and IBS are accompanied by significant extraintestinal co-morbidities, particularly those psychological in nature. The ability of peripheral inflammation to influence CNS function is not a new idea, and has roots back to early work completed using a model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced experimental colitis that results in altered food intake patterns in rats[14]. These and other findings set the stage for discovering the dynamic bi-directional relationship between the GI tract and the CNS, now referred to as the gut-brain axis (GBA). In the context of IBD and IBS, patients exhibit significant psychiatric co-morbidities at a much higher frequency than is observed in the general population. The Canadian Community Health Survey (CCHS), conducted in 2000 through 2001, highlighted that individuals who self-reported a physician's diagnosis of IBD for ≥6 months in duration experienced rates of depression that tripled what was observed in the general population[15]. Interesting findings from

Kurina et al. also support the idea that patients suffering from IBD experience significantly higher rates of depression and anxiety. In that study, the temporal sequence of events was also considered and it was determined that, in certain cases, the onset of the depression and anxiety appeared to precede the onset of Ulcerative Colitis (UC), while the relationship reversed in other patients[16]. From these findings, it is difficult to elucidate whether or not one condition has the ability to influence the onset of the other, as the underlying GI condition may have remained undiagnosed at the time of psychiatric diagnosis. Intuitively, the physical discomfort experienced by patients during symptomatic flares of IBD and IBS should be sufficient to influence mood and behavior in a negative fashion. However, one cannot rule out the possibility that there are other mechanisms, such as altered intestinal microbiota compositions, that drive these changes in mood and behavior.

Bugs and the Brain

With the aforementioned evidence suggesting that certain pathogenic bacteria have the ability to influence CNS function and host behavior[5], it stands to reason that psychiatric co-morbidities during IBD and IBS may actually be driven by the temporal instability of the microbiota in the gut. During the course of a typical inflammatory response to an infection in the gut, immune cell-derived cytokines influence the brain indirectly via the peripheral nervous system (PNS) and directly via leaky circumventricular organs to induce classical sickness behavior, a coordinated set of behavioral adaptations in a sick host[17], ultimately to deal with the

insult and rectify the situation. Interestingly, the behavioral changes associated with the acute response to *Campylobacter jejuni* challenge occur in the absence of overt inflammation locally in the gut or systemically[7]. These results highlight two important findings. First, the CNS has the ability to detect and respond to newly introduced pathogenic bacteria in the gut. Lastly, this detection, at least in an acute sense, appears to occur independently of immune activation, suggesting that an additional mechanism may explain this intriguing example of the complex gut-brain axis. However, these findings reflect the influence of a pathogenic bacterium and it stands to reason that a similar response may be observed during temporal instability of the normal intestinal microbiota. Despite not being directly investigated, it is possible that the presence of *Campylobacter jejuni* in the gut influenced a shift in the composition of the intestinal microbiota, which triggered the observed changes in the CNS. Using 454 pyrosequencing techniques, it has previously been demonstrated that oral challenge with *Citrobacter rodentium,* also previously shown to influence host behavior[18], correlates with significant alterations in both the luminal and adherent microbiota, with notable decreases in the abundance of the *Lactobacillus* group[19]. The idea of a disturbance in the intestinal microbiota influencing host behavior has significant clinical implications, since it provides a mechanistic link that may help to explain the observed temporal instability of the microbiota and the concomitant increase in psychiatric co-morbidities in IBD and IBS patients.

Our laboratory has recently shown that perturbation of the microbiota using oral non-absorbable antimicrobials (ATMs, **Table 1**) in healthy, adult mice results in transient but significant alterations in the composition of the intestinal microbiota (**Figure 1**)[20]. Specifically, ATM treatment produces significant increases in the proportions of Firmicutes and Actinobacteria populations, while decreasing γ-proteobacteria and Bacteroidetes populations when administered *ad libitum* for 7 days[20]. This specific mixture of antimicrobials has previously been shown to pass through the GI tract with little to no absorption across the epithelial barrier[21], ensuring that its effects are restricted to the gut lumen and its microbial contents. Concomitantly, the ATM-induced temporal instability of the microbiota was associated with an increase in exploratory behavior of mice in the light/dark preference and step-down latency tests. These results were characterized by an increase in time spent in and zone entries into the aversive zone of the light/dark preference test and a decrease in the latency to step down from an elevated platform[20], behaviorally reminiscent of fear inhibition[22]. When administered to germ-free mice of the same background, the ATM mixture failed to influence host behavior. To rule out a systemic effect of potentially absorbed ATM compounds, mice of the same background were injected intraperitoneally with a lower dose of the ATM cocktail for 7 days. As in germ-free mice, no changes in exploratory behavior were observed. These results strongly suggest that the observed behavioral changes are not a direct consequence of ATM exposure and that a

disturbance of the stable resident microbiota is the necessary stimulus for influencing behavior and CNS function.

The Limbic Circuit, BDNF and…the Microbiota?

The brain is an immensely complex collection of neurons, connections and chemicals that fuel a constant stream of communication and chatter within it. Capable of producing a wide range of emotional responses based on previous experiences and memories, the limbic circuitry of the brain is composed of a number of intimately connected brain nuclei, particularly the amygdala and hippocampal formation, which contain a number of important biochemical signalling molecules. As one such molecule, brain-derived neurotrophic factor (BDNF) helps to maintain functional neurons and direct the birth of new ones. Since the chemical basis of learning and memory is thought to involve the formation of new neurons and synaptic connections, BDNF is largely implicated in the process.

In addition to the observed changes in host behavior, perturbation of the microbiota is also associated with transient alterations in the expression of BDNF in the hippocampus and amygdala[20]. Specifically, perturbation results in an increase in hippocampal BDNF and a decrease in amygdala BDNF expression. In the hippocampus, increased BDNF plays an anxiolytic and antidepressant role through alterations in brain serotonin (5-HT) neurotransmission[23]. On the other hand, the amygdala is involved in the acquisition and expression of fear, possibly through the formation and retrieval of memories, respectively[22]. Increases in BDNF in the

amygdala have been linked to fear learning[24] and over-activation of the amygdala has also been linked to depression[25]. In the context of increased exploratory behavior and fear inhibition in animals treated with non-absorbable ATMs to perturb the microbiota, the aforementioned alterations in BDNF expression appear to correlate with the functional roles of these limbic nuclei. This supports the idea that a disturbance in the stable intestinal microbiota results in differential activation of specific brain regions that govern fear and coordinate host behavior. The extent to which additional brain regions, both limbic and non-limbic, are affected remains unknown.

Connecting the Gap

Given the introduced literature up until this point, it is clear that bacteria in the gut can influence the brain in such a way that it adapts to cope with an ongoing peripheral insult. This adaptation appears to take on the form of a shift in the behavioral phenotype of the host. How the stimulus of either a foreign bacteria or a shift in the stability of the commensal microbiota in the gut reaches the brain remains largely unknown. Clues that point to the nature of the mechanism can be found in the previously discussed literature, notably in the work involving *Campylobacter jejuni* and *Citrobacter rodentium* challenge. Within these two models, a rapid onset of anxiety-like behavior was observed 6-7 hours post-challenge, changes that occurred in the absence of any inflammatory cytokine response[6,18]. The induction of sickness behavior undoubtedly requires an upregulation of circulating

cytokines[17], so the fact that these behavioral adaptations persist without any discernible changes in cytokine profiles suggests that some other tangible form of communication between gut and brain must be involved. Along with the acute challenge involving both pathogens, a concomitant increase in *c-fos* was also observed in the sensory ganglia of an important peripheral nerve that supplies a large portion of the GI tract, the vagus nerve[18, 7].

The immediate early gene product, c-fos

The expression of critical transcription factors has been a popular approach for studying functional activation pathways, particularly in the CNS. *c-fos* is a cellular oncoprotein 314 amino acids in length, which is transcribed from the *FOS* gene, a member of the Immediate Early Gene (IEG) family. *c-fos* is rapidly and transiently expressed in response to various stimuli, such as growth factors, cytokines, neurotransmitters and stress[46]. *c-fos* proteins dimerize with additional IEG products, such as c-Jun, to form the Activator Protein-1 (AP-1) complex that activates downstream effects related to cell proliferation, differentiation and plasticity[47]. Although immediate, the expression of *c-fos* is transient and may fall below detectable levels within 15 minutes following translation[46], making it a challenging candidate for detection.

Phosphorylation of *c-fos* by extracellular-signal-regulated kinases (Erk) in response to extracellular stimuli further enhances its transcriptional activity. Phosphorylation of Serine-32 (Ser32) and Threonine-232 (Thr232) residues by

Erk1/2 increases protein stability and nuclear localization, respectively[48]. Phosphorylation of the Thr232 residue on *c-fos* also perpetuates the transcriptional activity of the protein, making it a useful candidate when attempting to identify functional *c-fos* protein isolated in its area of action, the nucleus. This also alleviates some of the concern regarding the longevity of *c-fos* expression, allowing for a more functional and stable target. The nuclear export mechanism of *c-fos* is reliant on the nuclear export system ($NES_{221-233}$) of amino acids 221-233 of the peptide chain[47]. Therefore, if phosphorylated at the Thr232 residue, the protein is spared from nuclear export and from degradation by cytoplasmic ubiquitin-protein ligase E3 component n-recognin (UBR).

Innervation of the Gut

The GI tract performs a number of involuntary and vital functions, including nutrient absorption, defence against pathogens and normal motility and propulsion of foodstuffs. The latter of which is coordinated by a highly complex network of nerve fibers that act as one to maintain proper GI health. The enteric nervous system (ENS) is a self-reinforcing neural network that maintains the ability to function and govern GI motility alone, but requires bi-directional communication with the CNS to function optimally[26]. Three major plexuses form what is considered to be the ENS, the myenteric, submucosal and mucosal plexuses. Together, these layers are considered to be the intrinsic innervation of the gut and their connecting association fibers form a network of nerves embedded within the majority of the length of the

gut wall (see Furness, 2006, **Figure 2**)[26]. In addition to the ENS, the autonomic nervous system (ANS) is composed of the parasympathetic nervous system (PSNS) and sympathetic nervous system (SNS). With respect to the GI tract, PSNS, SNS and spinal afferent nerve fibers are considered to be the extrinsic innervation that intimately associated with the ENS (see Furness et al., 1999, **Figure 3**)[27] and afford the necessary conduit to maintain the previously mentioned bi-directional communication with the CNS, primarily through intimate connections with the spinal cord. The vast innervation of the GI tract and the connections between intrinsic and extrinsic fibers allows the CNS to monitor a number of gut parameters, from chemical sensing in the lumen, to sensing mechanical stress along the gut wall.

Vagal Innervation of the Gut

The intimacy between the vagus nerve (cranial nerve X) and the GI tract could not be fully appreciated without the early efforts involving anterograde and retrograde labelling using fluorochromes, such as dextran 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). DiI is a lipophilic molecule that, once inserted into a cell, travels by lateral flow along the plasma membrane and can be visualized under the proper wavelength. Vagal anatomy and distribution within the GI tract has been elucidated by injecting DiI into the various motor (dorsal motor nucleus) and sensory (jugular and nodose ganglia) ganglia and following the fluorescent labelling[28,29]. Along the GI tract, the vagus nerve has three afferent endings within the gut wall: intraganglionic laminar endings (IGLEs) within the

myenteric plexus, intramuscular arrays (IMAs) within the smooth muscle layers and mucosal fibres within the mucosa[30]. The stomach maintains the highest density of vagal afferent endings, and this abundance tends to decrease as one moves rectally, with much less of a significant vagal representation in the distal regions of the GI tract[30,31].

Sympathetic Innervation of the Gut

The SNS represents the effector branch of the ANS responsible for suppressing GI functions during times of sympathetic activation. Having said that, SNS fibers share intimate associations with the ENS and gut tissue. Cell bodies of sympathetic neurons that supply specific aspects of the gut wall arise from the paravertebral sympathetic chain ganglia, located adjacent to the spinal column, as well as prevertebral (celiac and superior/inferior mesenteric) ganglia[32]. The destination of the axons from these ganglia is target specific, as paraverterbral sympathetic neurons almost exclusively innervate GI vasculature, whereas prevertebral neurons supply GI vasculature, control motility and sympathetic neurons that innervate secretomotor neurons in the gut wall (**Figure 4**)[32]. Axons from sympathetic neuron cell bodies extend to the gut mainly along the mesenteric nerves, but in upper thoracic regions extend with portions of the vagus nerve. They extend deep into the gut wall to the myenteric, submucosal and mucosal plexuses of the ENS[32] where they elicit their effector functions. The terminals of these axons are

composed of a number of neurotransmitters and their associated enzymes, mainly norepinephrine (NE) and tyrosine hydroxylase (TH), respectively.

Spinal Afferent Innervation of the Gut

The remaining contribution to the extrinsic innervation of the GI tract comes from spinal nerves that extend directly from the dorsal root ganglia (DRG) of the spinal cord. Unlike the extensive branching that characterizes the parasympathetic and sympathetic branches of the ANS previously discussed, spinal nerves that arise from the DRG of the spinal cord tend to extend to their GI targets with much less branching until they penetrate the gut wall and associate with the myenteric, submucosal, and mucosal plexuses. Axons that arise from the thoracic and lumbar DRG travel via the neighbouring sympathetic chain and extend to the gut along the splanchnic nerves, passing through the celiac and mesenteric ganglia to reach more proximal regions of the GI tract, while those axons that arise from sacral DRG extend to more distal regions of the GI tract via the parasympathetic pelvic nerves[33]. With respect to extrinsic spinal primary afferents supplying the distal colon, it has been demonstrated that specific DRG levels of the spinal cord are involved. By injecting the fluorescent retrograde neuronal tracer fast blue into the wall of the distal colon, DRG from T8-L1 and L6-S1 were shown to retain remnants of the initial dye[34], suggesting that spinal afferents arising from T8-L1 and L6-S1 were concerned with relaying sensory information from the distal colon, the region that harbours some of the densest bacterial populations found anywhere in the GI tract.

Bacteria-Gut-Brain Signalling

As illustrated in **Figure 2**, the gut wall is highly innervated[32]. Even the inner mucosa contains finite nerve endings that serve to sense and respond to mucosal and luminal contents, such as nutrients, immune cells and gut hormones. Evidence now suggests that these nerve endings may even respond to luminal bacteria, particularly pathogenic bacteria. The contraluminal application of NE enhances the luminal adherence of enterohemorrhagic *Escherichia coli* 0157:H7 (EHEC) in mouse cecal tissue in a dose-dependent manner[35]. Adherence of EHEC could be inhibited if the alpha-adrenergic-2 receptor antagonist phentolamine was applied prior to NE application, suggesting that sympathetic neurotransmitters can influence the adherence of pathogenic bacteria to the epithelium and that the nerve fibers associated with these adrenergic terminals may provide the necessary conduits for communication between the microbiota/gut and brain. The aforementioned findings of Goehler et al. provide more direct evidence that peripheral nerves may be involved in bacteria-related neurotransmission from gut to brain. Aside from the resultant behavioral changes observed following 6-7 hours of *Campylobacter jejuni*[18] and *Citrobacter rodentium*[6] exposure, a concomitant increase in *c-fos*, an important transcription factor involved in neuronal activation, was observed in the vagal sensory nodose ganglia of challenged animals. These results suggest that peripheral sensory nerves, particularly the parasympathetic vagus, may be responsible for the early, immune-independent, detection of pathogenic bacteria.

The administration of probiotic bacteria has also shed light on how particular bacteria communicate with the CNS. As defined by the World Health Organization, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[36]. *Bifidobacterium* species, particularly *Bifidobacterium longum*, have been investigated for such health benefits, and it is becoming clear that they can positively influence not only GI function, but CNS function as well. Oral administration of dextran sulphate sodium (DSS) in mice results in a low-grade inflammation of the GI tract, particularly the small intestine, characterized by mucosal and submucosal mononuclear cell infiltration[37]. The low-grade inflammation associated with DSS-treatment is accompanied by a vagal-dependent anxiety-like behavior, as surgical resection of the vagus nerve (vagotomy) attenuates the emergence of the behavioral phenotype[38]. Similarly, treatment with the probiotic *B. longum* NCC3001 (Nestle, Switzerland) normalizes DSS-induced anxiety-like behavior, without normalizing any of the up-regulated inflammatory markers associated with DSS administration. However, vagotomy also attenuates *B. longum's* normalizing effect on DSS-induced anxiety-like behavior, suggesting that the probiotic's anxiolytic effects are also vagally mediated from gut-to-brain[38]. Because *B. longum* itself does not elicit an inflammatory response, this is among the first lines of evidence to suggest that an autonomic pathway, the vagus nerve, is capable of relaying information about the presence of a novel bacterium from the gut-to-brain, albeit a beneficial bacterium.

The Big Picture

Our knowledge and understanding of the influence that the intestinal microbiota has on host development and function seems to grow by the day. Given the discussed literature, it seems evident that bacteria, whether pathogenic or beneficial, maintain the ability to exploit host physiology in order to communicate with the CNS, possibly through the PNS, although one cannot rule out a contribution from humoral metabolite-derived mechanisms. In addition to novel bacteria, the resident microbiota as a whole appears to act in a similar fashion and is capable of participating in a homeostatic relationship with the host, particularly the governing CNS. This communication ultimately appears to influence specific regions of the brain that govern the behavioral phenotype of the host, presumably in a fashion that will confer some type of benefit to the microbiota itself. Given the symbiotic evolution that has occurred between mammals and environmental bacteria, it is not surprising that such a relationship exists, since the intestinal microbiota has no less a stake in their survival in the relationship than does the host. Highlighting the particular regions involved in this microbiota-gut-brain axis, as well as the mechanism(s) involved, is extremely important in curbing our understanding of how the microbiota and CNS interact.

Hypothesis

The aim of this thesis project is to investigate regions of the brain that are responsive to disturbances in the stable intestinal microbiota, with a particular

emphasis on the regions that govern host behavior and ascending visceral stimuli. In addition to this, an investigation into the branches of the PNS that might be involved in providing a communicative conduit between the gut and the brain during microbiota-gut-brain axis signalling is also considered. This work tests the hypothesis that perturbation of the stable intestinal microbiota alters the pattern of neuronal activation in specific brain regions involved in coordinating host behavior and that this occurs in a peripherally neural-dependent mechanism. The specific project aims are to:

1. Reproduce intestinal microbiota-induced changes in behavior;
2. Assess the role of the vagus nerve in mediating microbiota-induced changes in behavior;
3. Develop a model of chemical sympathectomy using the selective noradrenergic neurotoxin 6-hydroxydopamine (6-OHDA);
4. Assess the role of the sympathetic nervous system in mediating microbiota-induced changes in behavior using chemical sympathectomy;
5. Investigate and characterize brain regions responsive to alterations in the intestinal microbiota, using immunohistochemistry.

MATERIALS AND METHODS

Animals

6-8 week old female BALB/c specific pathogen-free (SPF) mice were purchased from Harlan, Mississauga, Ontario, Canada and housed in a conventional specific pathogen-free (SPF) unit at McMaster University Central Animal Facility. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and received approval from the McMaster University Animal Research Ethics Board.

Surgical Vagotomy

Subdiaphragmatic vagotomy was performed in animals as previously described[39]. Briefly, after ketamine/xylazine anesthesia the ventral and dorsal truncal branches of the subdiaphragmatic vagus nerve were cut and a surgical pyloroplasty was performed. In sham-operated mice, vagal trunks were similarly exposed but not cut, and the pyloroplasty was performed. All mice were monitored daily for 1 week after surgery.

Chemical Sympathectomy

A group of mice underwent chemical sympathectomy, as described previously[40]. Briefly, mice received two intraperitoneal (IP) injections of the selective adrenergic neurotoxin 6-hydroxydopamine (100 mg/kg/body weight), while control mice received saline IP. The success of sympathectomy was confirmed

using immunofluorescent staining for the adrenergic nerve marker tyrosine hydroxylase (TH), the rate limiting enzyme in NE synthesis.

Antimicrobial Treatment

One group of mice (n=14) received a mixture of non-absorbable antimicrobials (bacitracin and neomycin, 5ug/mL; pimaricin, 5uL/mL, Sigma Aldrich, Canada) *ad libitum* in sterile drinking water for 7 days, as previously described (Bercik et al., 2011). Control animals (n=14) continued receiving sterile drinking water only. All mice were monitored and weighed daily. On Day 7, all animals underwent behavior testing and were sacrificed for retrieval of tissues of interest.

Behavior

Animal behavior was assessed in mice using the light/dark preference test as described[41] using an automated detection system (Med Associates Inc, St. Albans, Vermont, USA). Briefly, individual mice were placed in the center of the "aversive" zone lit by ambient light that was attached via a small doorway to a smaller "safe" dark box in the same arena and animal behavior was assessed for 10 minutes. Measurements considered included: total time spent in the light region, number of entries into the light region, total vertical/rearing counts, total distance traveled, average velocity and total resting time. Quantification was performed by an automated computer program (Med Associates Inc, St. Albans, Vermont, USA). Additionally, exploratory behavior was assessed using the step-down latency test[42].

Briefly, individual mice were placed on the center of an elevated platform (diameter, 10cm; height, 4cm) and the time in which it took the mouse to step down and vacate the platform using both two paws and all four paws was measured (maximum time, 5 minutes). Time to step down was recorded and analyzed according to the statistical analysis section.

Tissue Processing

Upon sacrifice of the animal, brain and colon tissue were rapidly removed, fixed in 10% formalin for 48 hours, cryo-protected in graded sucrose (10, 20 and 30%) and then gradually frozen in an embedding medium (Tissue-Tek, CA, USA) over dry ice, and stored at -20°C. All tissues were then cut into 10µm sections on a Microm HM 550 cryostat (Thermo Scientific, WI, USA) and mounted onto pre-cleaned doublefrost slides (Surgipath, Manitoba, Canada) and stored at -20°C until processing. Brains were sectioned at regions of interest (ROI) using The Mouse Brain Atlas of Paxinos and Franklin, 2001[43]. ROIs include: dentate gyrus (DG, bregma -1.58) hippocampus (two sections at bregma -1.58mm and -2.70mm), and the nucleus of the solitary tract (NTS, one section at bregma -6.72). Serial sections of 10µm approximately one millimetre apart were sections from colon tissues, while entire ganglia were sectioned at 10µm on mounted onto slides.

Immunohistochemistry

Tissues were allowed to thaw at room temperature for one hour then washed in phosphate-buffered saline (PBS, pH 7.4) for 5 minutes to remove any residual OCT. Tissue sections were fixed in 10% formalin for 20 minutes, and endogenous peroxidases (brain only) and proteins were blocked using 0.3% hydrogen peroxide and 10% normal serum (containing 0.4% Triton-X-100) for 15 and 20 minutes, respectively. Endogenous avidin and biotin was also serially blocked (brain only, Vector, Canada). Primary antibodies (**Table 4**) to *c-fos, Thr-232-c-fos* and *TH* were applied overnight in a humidity chamber for 12-18 hours at 4°C, and then detected with the appropriate secondary antibodies (**Table 5**) for 90 minutes at 4°C. In brain sections, antibody-labelled cells were then visualized using the ABC Elite Kit (Vector, Canada) and VIP Substrate (Vector, Canada). Stained sections were coverslipped using aqueous mounting media (Vector, Canada).

Image Analysis

Colon tissue sections were viewed with an Olympus BX51 fluorescent microscope (Olympus, Canada), and images were captured using a QImaging Micropublisher 3.3 RTV camera (QImaging, BC, Canada) and Image Pro Plus 6.3 imaging software (MediaCybernetics, MD, USA). Brain tissue sections were viewed with a Leica DMLS microscope (Leica Microsystems, Wetzlar, Germany), and images were captured using an Olympus QColor 3 camera (Olympus, Canada) and Adobe Photoshop CS2 imaging software (Adobe System, USA). Positively stained cells were

measured using ImageJ analysis software (NIH). Briefly, particle measurements, in tandem with set color thresholds, were used to quantify the number of *c-fos* and *Thr-232-c-fos* immuno-positive cells. Positive cell counts were analyzed using Prism 4 software (GraphPad Software, Inc, CA, USA).

Statistical Analysis

All data are presented as means ± standard deviation. Behavioral data were analyzed by non-paired student t-test and one-way ANOVA with bonferroni post-hoc analysis where appropriate with Prism 4 software. Immunohistochemistry was analyzed using a non-paired student t-test with Prism 4 software. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Systemic IP injections of 6-OHDA significantly reduces TH expression in the distal colon

The systemic administration of 6-OHDA significantly reduced the expression of TH in the distal colon of treated animals (**Figure 5**), relative to controls (controls, 100%±2.99, n=14; 6-OHDA, 7.86%±1.54, n=14, **Figure 6**).

ATM-induced perturbation of the microbiota influences host behavior

ATM-treated animals spent significantly more time in the aversive light zone of the light/dark preference test than did controls, while there was no difference between the two groups with respect to total distance traveled in both light and dark zones (**Figure 7**, **Table 2**). ATM-treated animals also had a significantly higher number of entries into the aversive light zone, as well as rearing counts (**Figure 7**, **Table 2**). Other motor parameters measured, including average velocity and total resting time, were not significantly different between groups (**Figure 7**, **Table 2**). ATM-treated animals also displayed a reduced latency to step down from an elevated platform, whereas the majority of control animals failed to step down during the 5 minute trial (**Figure 8**, **Table 2**).

Surgical vagotomy fails to attenuate ATM-induced behavioral changes

Surgical vagotomy alone does not influence host behavior in the light/dark preference or step-down latency tests, as indicated by no difference in measured parameters compared to control mice (**Figures 8 and 9**, **Table 2**). When surgical

vagotomy is paired with ATM treatment, time to step down with either two or four paws is not significantly different relative to ATM treatment alone (**Figures 8**, **Table 2**). In the light/dark preference test, apprehensive parameters values for vagotomised mice (time in light, entries into light) are not statistically different from ATM treatment values, while motor parameters values are comparable among all groups (**Figure 9**, **Table 2**).

Chemical sympathectomy fails to attenuate ATM-induced behavioral changes

Chemical sympathectomy alone did not influence host behavior in the light/dark preference or step-down latency tests, as indicated by no difference in measured parameters compared to control mice (**Figure 10**, **Table 2**). When chemical sympathectomy is paired with ATM treatment, time to step down with either two or four paws was not significantly different relative to ATM treatment alone (**Figures 8**, **Table 2**). In the light/dark preference test, apprehensive parameters values for sympathectomized mice (time in light, entries into light) were not statistically different from ATM treatment values, while motor parameters (total distance, average velocity) values were comparable among all groups (**Figure 10**, **Table 2**).

Perturbation of the microbiota produces altered patterns of neuronal c-fos in specific brain regions

On day 7 of ATM treatment, animals were sacrificed and brain tissue was harvested and prepared for immunohistochemistry. The hippocampal formation, composed of the hippocampal cell layer and dentate gyrus (DG), and nucleus of the solitary tract

(NTS) were analyzed for c-fos staining using the methods previously described. In the DG, the expression of c-fos and protein was significantly enhanced in ATM-treated mice in comparison to controls (**Figure 11**, **Table 3**). In this region, the expression of phosphorylated c-fos protein was also significantly enhanced in ATM-treated animals (**Figure 12**, **Table 3**). In the hippocampal cell layer regions (CA1, CA2, CA3), the expression of c-fos was significantly enhanced in ATM-treated mice in comparison to controls (**Figure 13**, **Table 3**). In this region, the expression of phosphorylated c-fos protein was also significantly enhanced in ATM-treated animals (**Figure 14**, **Table 3**). In the NTS, there was no difference in the expression of either c-fos or phosphorylated c-fos proteins between groups (**Figure 15**, **Table 3**).

DISCUSSION

This project tested the hypothesis that perturbation of the stable intestinal microbiota alters the pattern of neuronal activation in specific brain regions involved in coordinating host behavior and that this occurs in a PNS-dependent mechanism. A mixture of non-absorbable antimicrobials was administered orally to BALB/c mice over the course of 7 days in order to significantly destabilize the intestinal microbiota. In addition to a shift in microbiota composition, these mice displayed an increase in exploratory behavior, suggestive of inhibition of the innate fear response. Altered host behavior as a result of microbial perturbation in the gut correlates with an increase in the expression of neuronal *c-fos* and *Thr-232-c-fos* in the hippocampal formation and occurs independently of vagal-parasympathetic and sympathetic nerve transmission.

A microbiota-dependent effect

The oral administration of an antimicrobial mixture of bacitracin, neomycin and pimaricin compounds has previously been shown to have negligible absorption[21] and be an effective strategy for specifically disturbing the intestinal microbiota[20]. It has been demonstrated that chronic, low-grade inflammation is a sufficient stimulus to influence CNS function[44] and it is worth mentioning that neomycin has been shown to enhance the migration of macrophages into the lamina propria[45]. However, one would expect neomycin toxicity, or other antimicrobial compounds, to result in a sickness-like behavioral phenotype characterized by

general malaise and a decrease in motor activity. Given the results of this project, there were no observed differences in motor parameters during behavioral testing between control and ATM-treated animals, suggesting that direct stimulatory effects of the administered ATMs on colonic tissue are not likely to account for the observed behavioral changes. Two important lines of additional evidence are worth highlighting that reiterate this point. First, low-dose IP injections of the same ATM mixture fail to produce any discernible increase in exploratory behavior in treated mice[20], suggesting that the observed behavioral phenotype is not a direct result of ATM compounds acting on the CNS. Second, the same antimicrobial mixture and regimen has been administered to germ-free mice, which lack an endogenous microbiota. Unlike the behavioral changes observed in the SPF mice in this project, germ-free ATM-treated mice fail to display an increase in exploratory behavior in the light/dark preference and step-down latency tests[20]. These findings strengthen the notion that this effect is indeed a microbiota-dependent effect and does not result from direct ATM interaction with the CNS.

Commensal Influence on CNS function

Both the hippocampus and DG are major components of the mammalian limbic system, a collection of intimately connected structures in the brain that govern a wide variety of processes, namely in relation to emotions and behavior. While the DG is widely believed to contribute to the birth of new neurons, a process referred to as neurogenesis, the hippocampus is thought to be concerned with

learning and memory. These structures, and the processes they govern, are highly susceptible to both internal and external stimuli, and the microbiota is no exception.

Dietary-induced shifts in microbial diversity in the gut have been correlated with enhanced learning and working memory capacity in mice[49], suggesting that dietary influences on behavior and memory may actually comprise a strong microbial component. The findings from the current project fit well with these observations, as a shift in microbiota diversity as a direct result of perturbation using oral ATMs in the gut enhances the expression of an important transcription factor in regions of the brain known to govern behavior and memory. Additional evidence for an influence of the microbiota on limbic system functioning was demonstrated using the same ATM mixture and subsequent alterations in the expression of BDNF. Oral ATM treatment enhances BDNF expression in the hippocampus, while decreasing its expression in the amygdala[20]. An increase in the expression of both *c-fos* and *Thr-232-c-fos* in the hippocampus correlates with the findings relating to increased hippocampal BDNF expression and exploratory behavior in mice. An increase in both *c-fos* and *Thr-232-c-fos* in the dentate gyrus specifically is a novel finding, one that will require further investigation in order to determine the functional role of this effect. The amygdala is another important structure of the limbic system that governs the expression of fear, particularly the acquisition and retention of fear memories. Given the increase in exploratory behavior and apparent inhibition of fear following perturbation of the microbiota, a

decrease in amygdala-derived BDNF seems logical and suggests that disturbing the stable microbiota inhibits certain aspects of amygdalar function. This may involve *N*-methyl-D-aspartate (NMDA) receptors within the amygdala, as direct NMDA receptor antagonism has been shown to inhibit the initial acquisition of fear memories in rats[50]. It is also possible during episodes of perturbation that commensal microbes signal the brain using synthesized receptor ligands that influence the function of specific regions, such as the amygdala and hippocampus. One such receptor complex highly implicated in fear and anxiety is the benzodiazepine (BDZ)/$GABA_A$ receptor complex, which has been shown to be differentially expressed in Balb/c and C57BL/6 mice, two strains with completely different patterns of fear expression[51]. Both BDZ and $GABA_A$ receptors have a high affinity for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS. It has been suggested that the commensal microbiota is capable of producing endogenous BDZ/$GABA_A$ receptor ligands[52], so in a scenario of perturbation where perhaps an ATM-resistant bacterium who produces such a ligand (eg. GABA-like substrate) is offered the environment to reproduce and grow, it is conceivable that this may influence the inhibition of specific brain regions (eg. amygdala) and represent a potential mechanism of altering Balb/c behavior and fear. Decreased BDNF expression in the amygdala following perturbation of the microbiota[20] supports this idea. Investigating *c-fos* expression in the various amygdalar nuclei is important and should be considered in future experiments in order to separate the different functions associated with different amygdalar nuclei.

In addition to the amygdala, responses to alterations in the distant microbiota should be investigated in other brain nuclei to improve our understanding of the relationship between commensal bacteria and the CNS.

Mechanisms

Although complex, the nature of this microbiota-gut-brain signalling is likely to take on one of two forms and may perhaps involve a combination of both. The first of which involves neural signalling from gut-to-brain via PNS pathways. Aforementioned evidence introduced the apparent interaction between pathogenic EHEC and the sympathetic neurotransmitter NE, which could be blocked by the administration of adrenergic receptor antagonist phentolamine[53], suggesting that perhaps bacterial communication between the gut and CNS requires sympathetic neurotransmission. In the current project, behavioral changes as a result of perturbation of the microbiota also persisted during chemical sympathectomy using the selective adrenergic neurotoxin 6-OHDA (**Figure 5**), suggesting that commensals do not exploit sympathetic neurotransmission as a means to communicate with the CNS. Much evidence has implicated a role for parasympathetic-vagal signalling during the introduction of pathogenic[18,6] and probiotic bacteria[38]. Given the extensive contributions of the vagus to GI innervation, particularly in the upper regions, this is not surprising and may represent a type of detection system for novel bacteria, particularly pathogenic, that enter the GI environment. Be that as it may, vagal integrity was not important in the context of microbiota-gut-brain signalling,

as evidenced by a persistent behavioral change following perturbation of the commensal microbiota with surgical vagotomy. The current project also took into account functional changes within the NTS. Located in the brainstem, the NTS is the primary visceral relay center, representing the primary sensory nucleus for both taste and GI visceral sensation[54]. The NTS receives heavy ascending peripheral input from the vagus nerve, the spinoreticular tract and circulatory matter by way of a leaky circumventricular organ, the area postrema. The expression patterns of *c-fos* and in this nucleus did not differ between groups, suggesting that either the NTS is not involved in the ascending microbiota-gut-brain signalling or that *c-fos* itself is not involved in this aspect of NTS neurotransmission.

Given that vagal-parasympathetic and sympathetic pathways do not appear to be involved in microbiota-gut-brain signalling, spinal nerves represent the remaining peripheral pathway that constitutes the extrinsic innervation of the gut. Surgical resection of these nerves or dissection and removal of their associated DRG[34] following perturbation may highlight whether or not they are involved in this paradigm of microbiota-gut-brain signalling.

The second potential mechanism involves accessing the brain via humoral metabolites crossing the blood-brain barrier, either directly produced by bacteria themselves or indirectly by a bacterial influence on physiology. Broad metabolomic comparisons between SPF and germ-free mice have revealed striking differences in metabolic phenotypes[55], providing a possible explanation for how commensals, during times of instability, can influence the function of systems outside of the GI

tract. Perturbation of the microbiota using the antibiotic vancomycin results in transient fluctuations in both microbiota diversity and the metabolic profiles of urine and fecal samples, namely hippurate, di- and trimethylamine, oligosaccharides and choline[56]. Specifically, there was an observed increase in circulating levels of choline in vancomycin-treated animals. This is an intriguing finding, as choline supplementation in rats has been shown to significantly influence the development of the hippocampus and enhance memory[57]. Although memory was not tested in the current project, these findings correlate with an increase in *c-fos* expression in the hippocampal formation, since this region of the brain is highly involved in the formation and retrieval of memories. Whether or not an increase in hippocampal activation was a consequence of changes in circulating metabolites as a result of perturbation of the microbiota is unknown. The expression of BDNF in cultured neuroblastoma cells is unaffected when exposed to serum from *B. longum* treated mice[38], suggesting that this probiotic does not influence the brain in a metabolite-dependent mechanism. Whether or not the commensal microbiota bypasses humoral mechanisms and exploits PNS pathways as a means to signal the brain remains unknown.

Conclusions

In the current project, perturbation of the microbiota using oral, non-absorbable antimicrobials results in an increase in exploratory behavior. This effect correlates with an increase in both *c-fos* and *Thr-232-c-fos* in the hippocampus and

DG, suggesting that the increase in exploratory behavior represents activation of brain circuitry that involves at least these two regions. These results also suggest that the brain retains the ability to monitor and remain informed about the composition of the intestinal microbiota and respond to temporal alterations by modifying the behavior of the host. This microbiota-gut-brain signalling is independent of vagal-parasympathetic and sympathetic neurotransmission. These findings highlight a key difference between the gut-brain signalling mechanisms employed by pathogenic/probiotic and commensal bacteria within the gut that has previously gone unappreciated.

Table 1. Composition of the orally administered ATM mixture

Antibiotic Compound	**Concentration**	**Target**
Bacitracin	5mg/mL	Gram-positive cell walls (++)
Neomycin	5mg/mL	Gram-positive cell walls (+) Gram-negative (+++)
Pimaricin	5uL/mL	Antifungal agent (yeast overgrowth)

For a more detailed investigation into the composition and dose-effects of this specific mixture of antimicrobial compounds, see van der Waaij D, Berghuis-de Vries JM, Korthals Altes C. Oral dose and faecal concentration of antibiotics during antibiotic decontamination in mice and in a patient. *J Hyg (Lond)*. 1974;73(2):197-203.

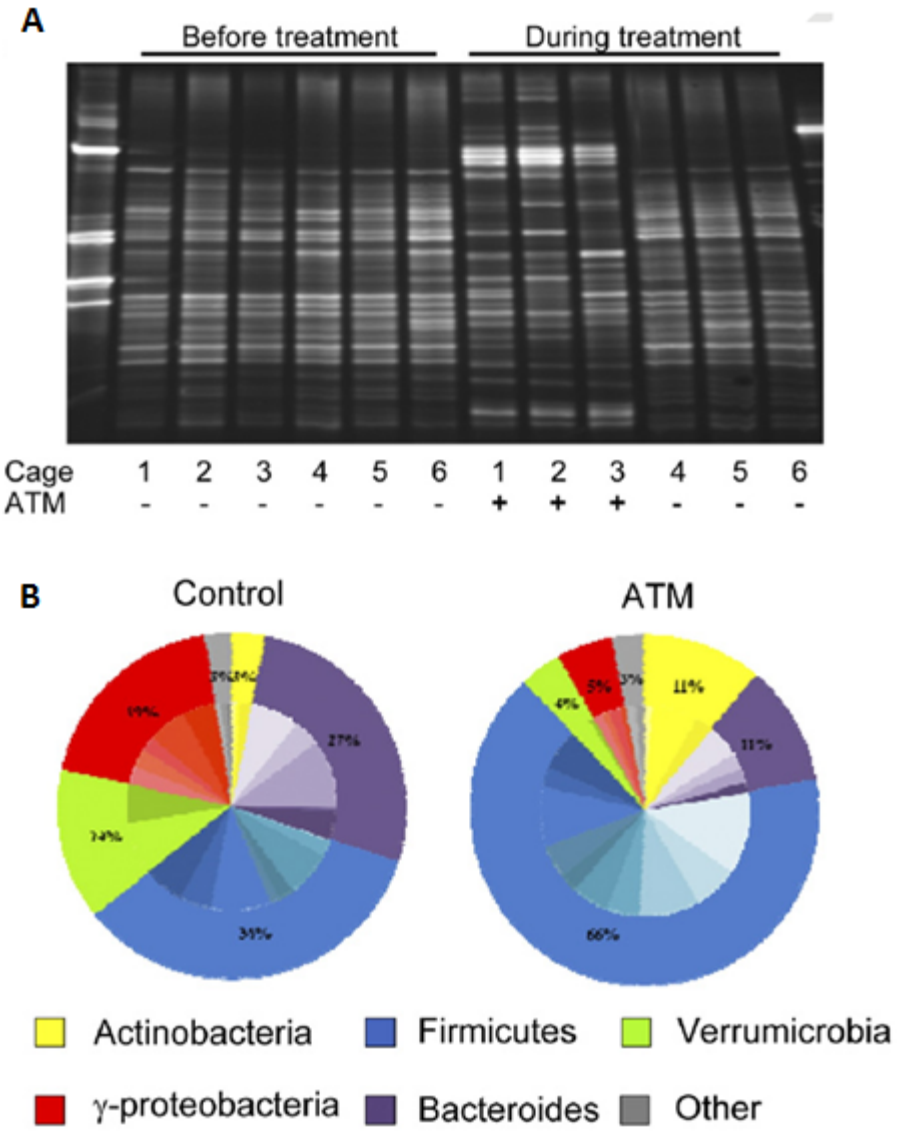


Figure 1. ATM-induced changes in microbiota. A) Denaturing gradient gel electrophoresis (DGGE) gel of fecal-derived microbiota DNA samples in control and ATM-treated mice before and after treatment. Samples were pooled from each cage (5 animals per cage). Cages 1-3 show marked alterations in the community profiles on day 7 of ATM treatment in comparison to controls who received only sterile water. B) Detailed analysis of microbiota by sequencing individual DNA bands (Figure credit, Bercik et al., *J Gastroenterology*, 2011)[20].

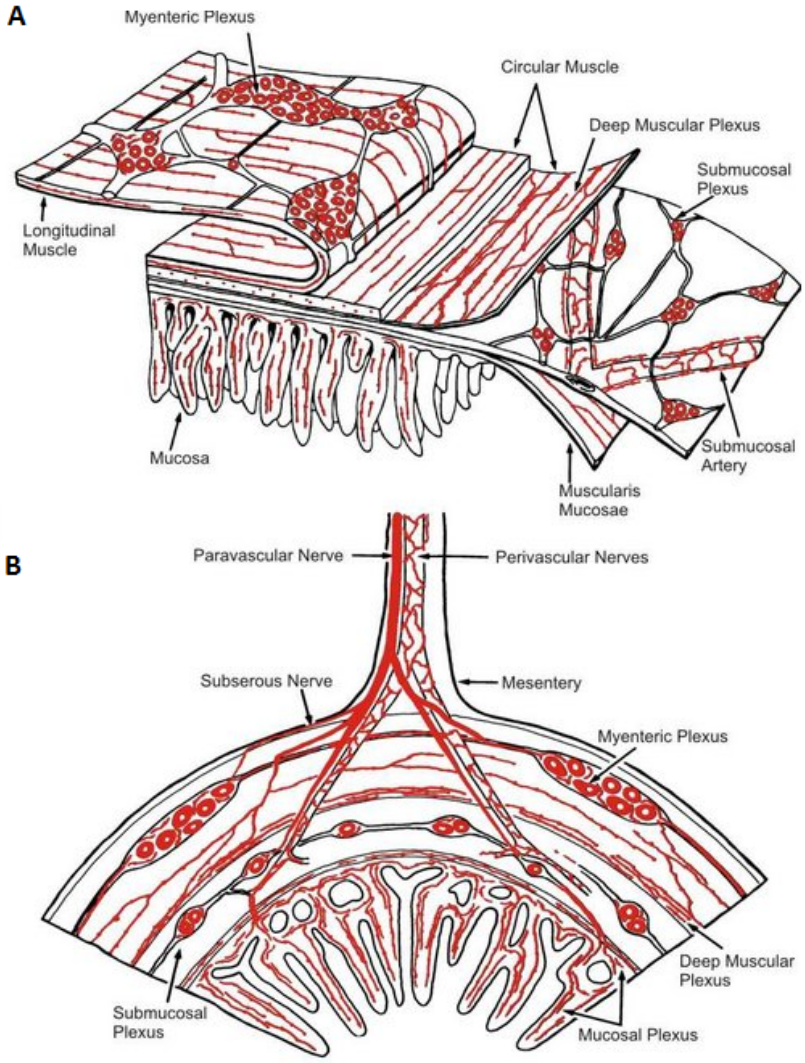


Figure 2. Cross-sectional structure of the ENS. A) Whole-mount highlighting the layers of the enteric nervous system embedded between muscular layers of the gut wall. B) Cross-sectional view of the different plexi in the gut wall. The outer myenteric plexus and middle submucosal plexus are both composed of connected ganglia, and are themselves connected. The inner mucosal plexus is composed of a dense network of finer nerve fibers that penetrate the lamina propria (Figure credit, Furness JB, *Blackwell*, 2006)[26].

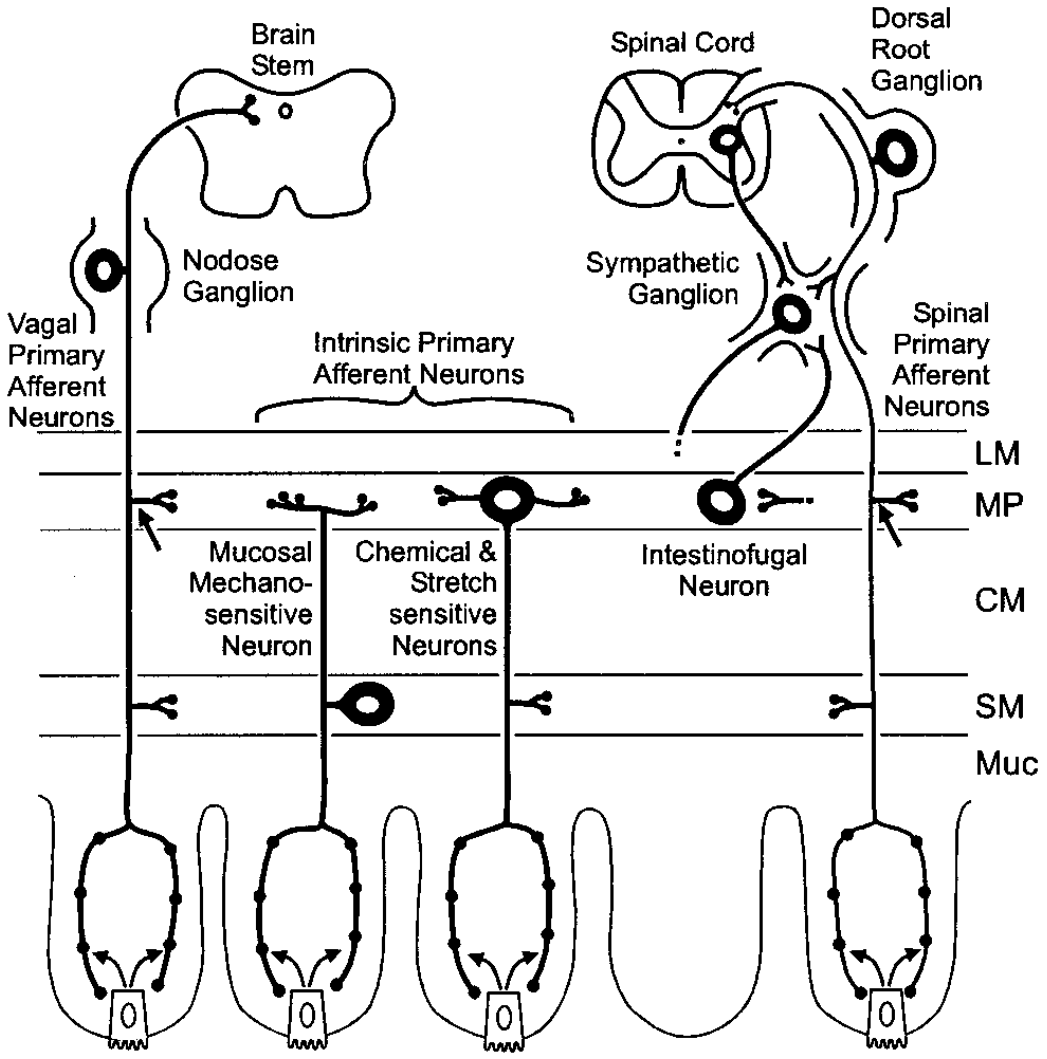


Figure 3. Intrinsic and extrinsic innervation of the gut wall. Parasympathetic, sympathetic and spinal afferent nerves provide the extrinsic innervation of the gut. Vagal primary afferents neurons synapse at cell bodies in the sensory nodose ganglia and NTS of the brainstem and also connect intimately with intrinsic primary afferents neurons (IPANs) that are confined to the gut wall. Sympathetic fibers synapse at peripheral sympathetic ganglia and the spinal cord. Spinal afferents have cells bodies in the dorsal root ganglia (DRG) just outside of the spinal cord (Figure credit, Furness et al., *Am J Physiol*, 1999)[27].

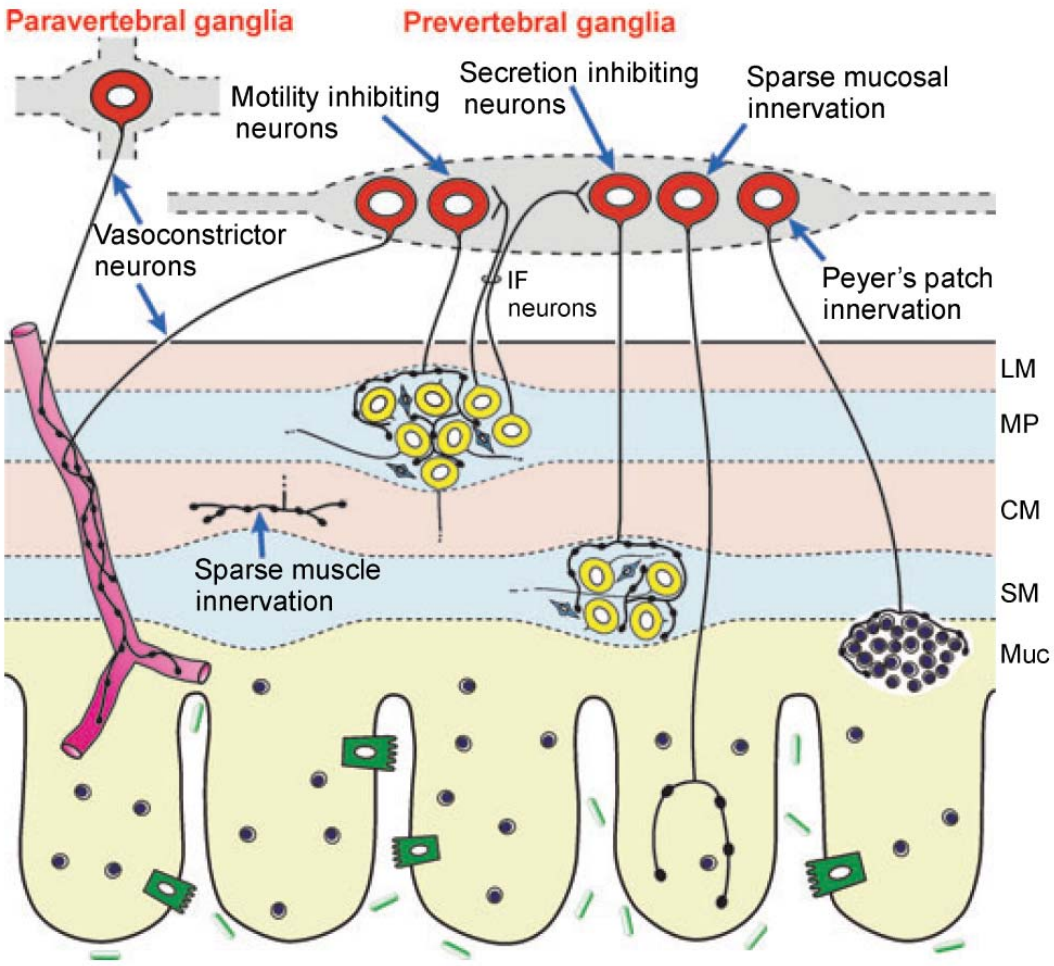


Figure 4. Illustration of the sympathetic branch of the extrinsic innervation of the intestine. Cells bodies of the sympathetic effector system can be found in both the sympathetic chain paraverterbral and preverterbral ganglia outside of the gut wall. Extending nerve fibers target specific regions and tissues within the gut wall. All levels of the ENS (myenteric, submucosal and mucosal plexuses) have connections with sympathetic fibers. Given this intimacy, sympathetic nerves are good candidates to investigate for microbiota-gut-brain signalling (figure credit, Lomax et al., *Neurogastroenterology and Motility*, 2010)[32].

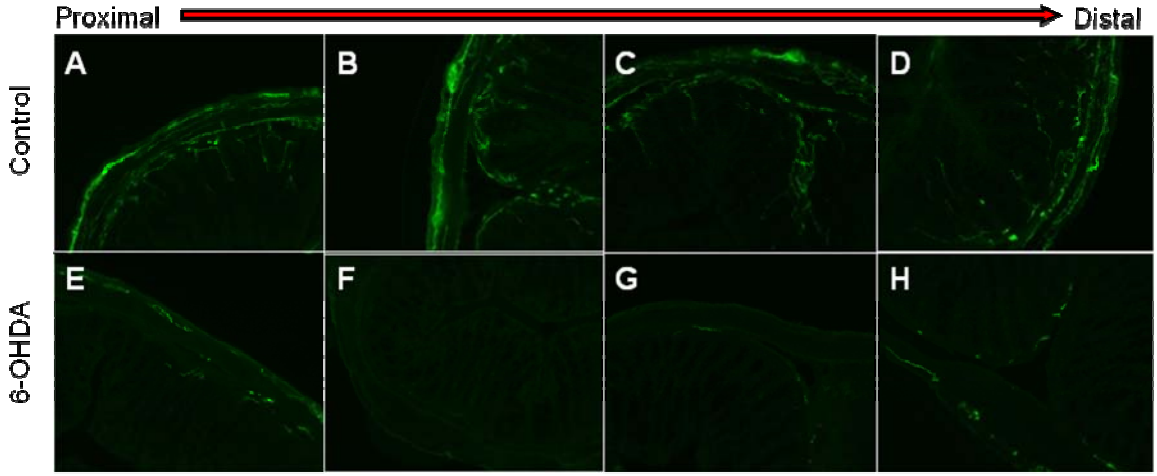


Figure 5. Verification of 6-OHDA-induced chemical sympathectomy using immunofluorescent staining of the adrenergic nerve marker TH in the distal colon. Animals received two IP injections separated by 16 hours of either sterile water (vehicle, n=14, A-D) or 6-OHDA (100 mg/kg/body weight, n=14, E-H). Three weeks later, animals were sacrificed and distal colon sections were stained for TH.

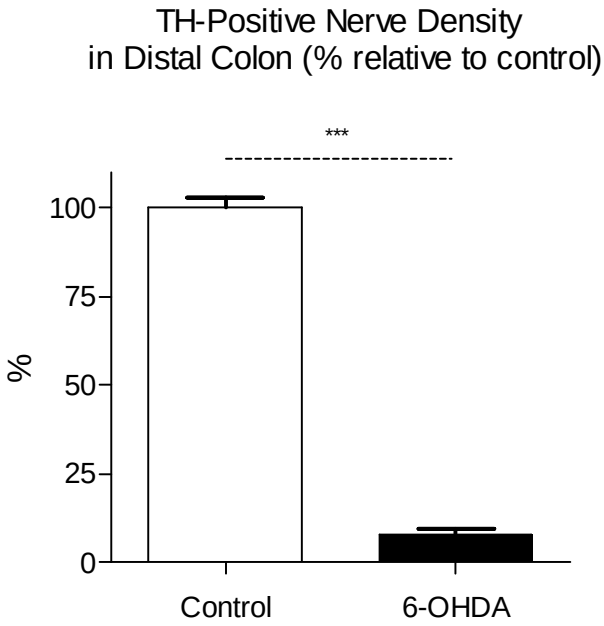


Figure 6. Quantification of TH-positive adrenergic nerve density in distal colon sections of water or 6-OHDA-treated mice. Sections were analyzed using ImageJ software using color thresholds and percent area measurements. ***p<0.001.

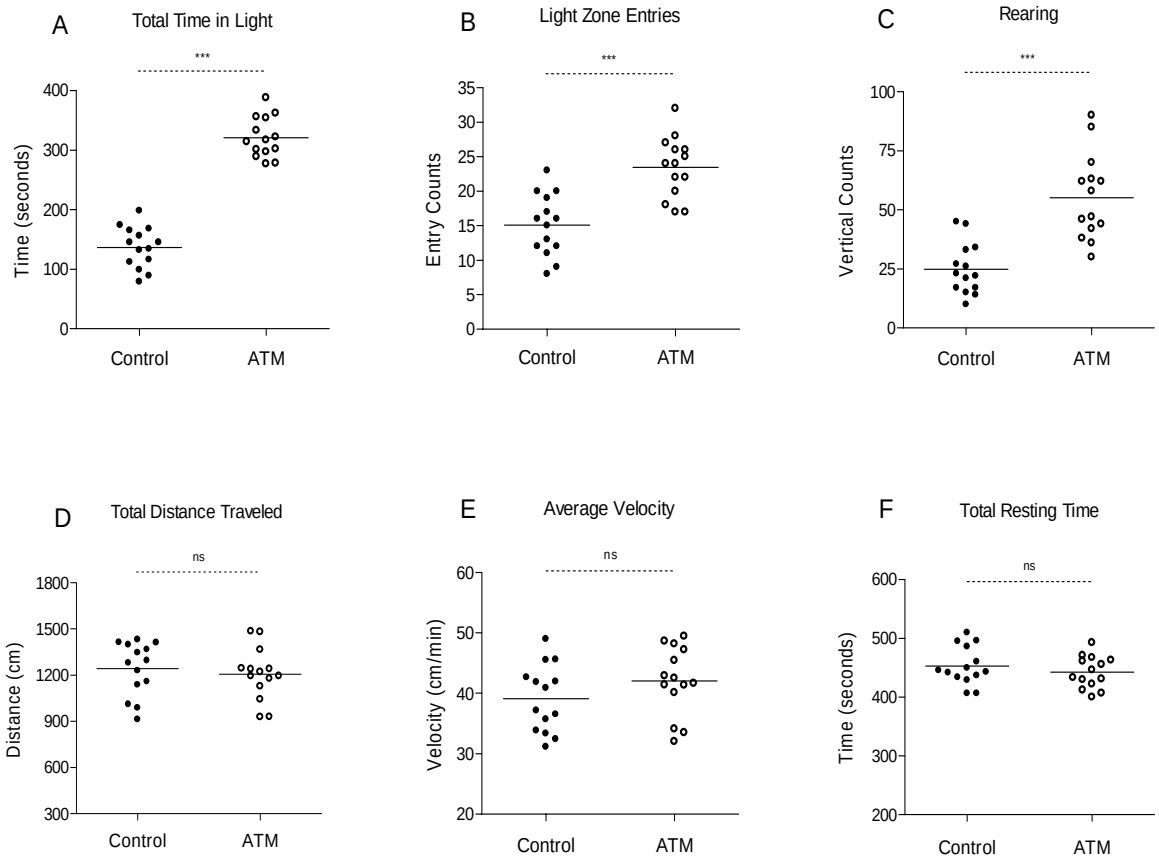


Figure 7. Oral ATM treatment promotes exploratory behavior without influencing motor parameters in the light/dark preference test. Results of the light/dark preference test in ATM-treated (n=14) and control (n=14) mice. Total time spent in the light zone (A), number of entries into the light zone (B), vertical rearing counts (C), total distance traveled (D), average velocity (E) and total resting time (F) were measured over a 10 minute time frame per mouse. ***, $p<0.0001$.

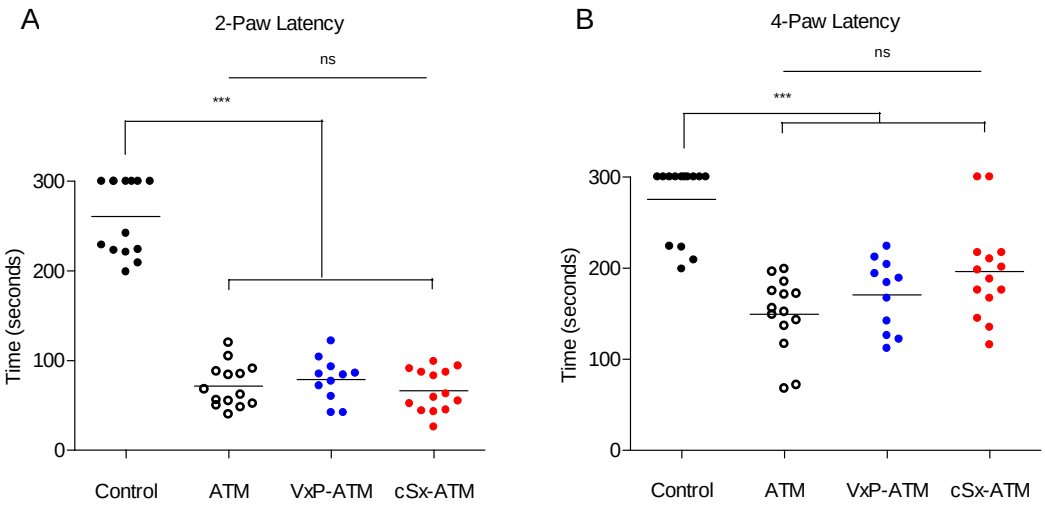


Figure 8. Oral ATM treatment promotes exploratory behavior in the step-down latency test. Results of the step-down latency test in control (n=14), ATM-treated (n=14), vagotomised (VxP, n=11) and sympathectomized (cSx, n=14) mice. The duration of time passed before animals stepped down with either two paws (A) or all four paws (B) was measured over a five minute period. ***, $p<0.001$.

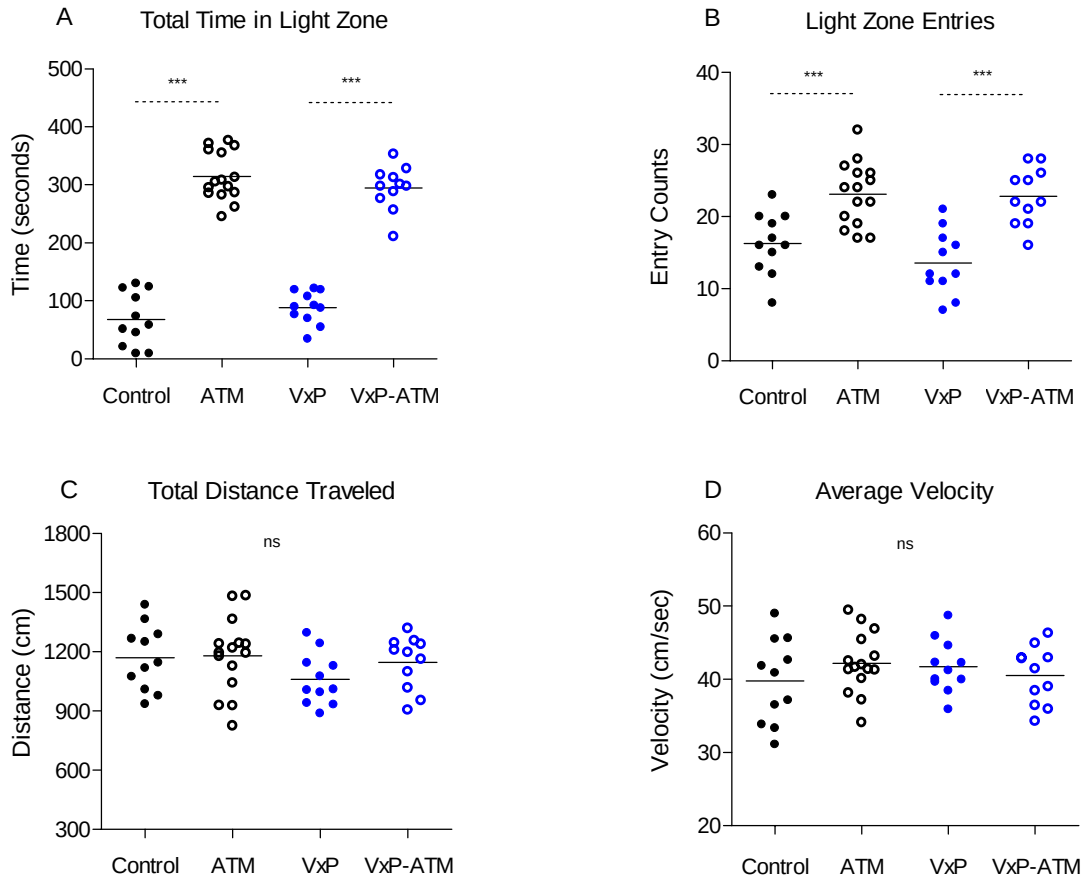


Figure 9. Surgical vagotomy fails to attenuate ATM-induced behavioral changes in the light/dark preference test. Results from the light/dark preference test in ATM-treated and control mice, with or without surgical vagotomy (VxP). Total time spent in the light zone (A), number of entries into the light zone (B), total distance traveled (C) and average velocity (D) were measured over a 10 minute time frame per mouse. ***, $p<0.001$; ns, no significance.

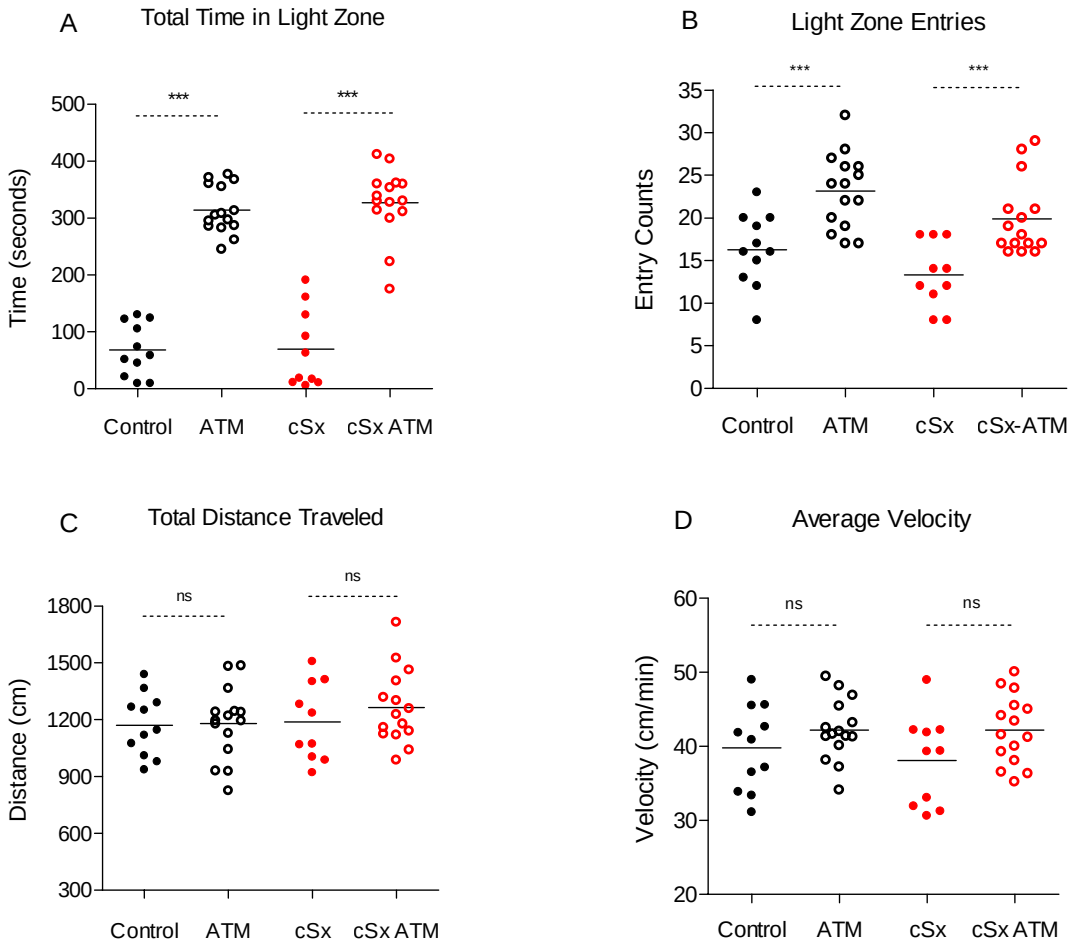


Figure 10. Chemical sympathectomy fails to attenuate ATM-induced behavioral changes in the light/dark preference test. Results from the light/dark preference test in ATM-treated mice, with or without chemical sympathectomy (cSx). Total time spent in the light zone (A), number of entries into the light zone (B), total distance traveled (C) and average velocity (D) were measured over a 10 minute time frame per mouse. ***, $p<0.001$; ns, no significance.

Table 2. Raw Data for light/dark preference and step down latency tests. Data presented as means ± standard deviation, t-test or ANOVA F values and degrees of freedom (df).

Test Condition / Group ID	Time in Light	Light Zone Entries	Rearing	Total Distance Traveled
Control	68.27±13.94 t=12.49 df= 26,	16.27±1.280 t=4.991 df=26	24.86±2.884 t=5.404 df=26	1170±49.68 t=0.5529 df=26
ATM	314.2±10.87 t=12.49 df=26	23.13±1.133 t=4.991 df=26	55.21±4.821 t=5.404 df=26	1180±49.15 t=0.5529 df=26
VxP	88.49±8.497 F=133.4 df=47	13.55±1.337 F=15.06 df=47	18.44±6.774 F=27.49 df=47	1060±39.46 F=1.349 df=47
VxP-ATM	294.6±11.36 F=133.4 df=47	22.82±1.182 F= 15.06 df=47	49.25±8.324 F=27.49 df=47	1146±40.59 F=1.349 df=47
cSx	69.99±22.01 F=133.4 df=47	13.30±1.212 F=15.06 df=47	21.41±4.954 F=27.49 df=47	1189±65.21 F=1.349 df=47
cSx-ATM	326.8±15.78 F=133.4 df=47	19.87±1.138 F=15.06 df=47	52.33±11.93 F=27.49 df=47	1264±50.64 F=1.349 df=47

Table 2. Cont'd

Test Condition / Group ID	Average Velocity	Total Resting Time	2-Paw Latency	4-Paw Latency
Control	39.77±1.746 t=1.376 df=26	452.9±8.724 t=0.9249 df=26	260.5±11.24 t=11.51 df=26	275.4±10.92 t=7.044 df=26
ATM	42.20±1.058 t=1.376 df=26	442.4±7.306 t=0.9249 df=26	71.71±6.405 t=11.51 df=26	149.4±10.86 t=7.044 df=26
VxP	41.74±1.090 F=0.7829 df=47	460.7±7.442 F=1.067 df=47	249.46±8.442 F=138.2 df=52	269.8±14.66 F=21.36 df=52
VxP-ATM	40.51±1.185 F=0.7829 df=47	445.2±11.45 F=1.067 df=47	78.82±7.343 F=138.2 df=52	170.5±11.82 F=21.36 df=52
cSx	38.08±1.933 F=0.7829 df=47	469.8±9.921 F=1.067 df=47	275.75±9.632 F=138.2 df=52	286.5±12.43 F=21.36 df=52
cSx-ATM	42.18±1.204 F=0.7829 df=47	443.2±7.778 F=1.067 df=47	66.29±6.244 F=138.2 df=52	196.1±14.29 F=21.36 df=52

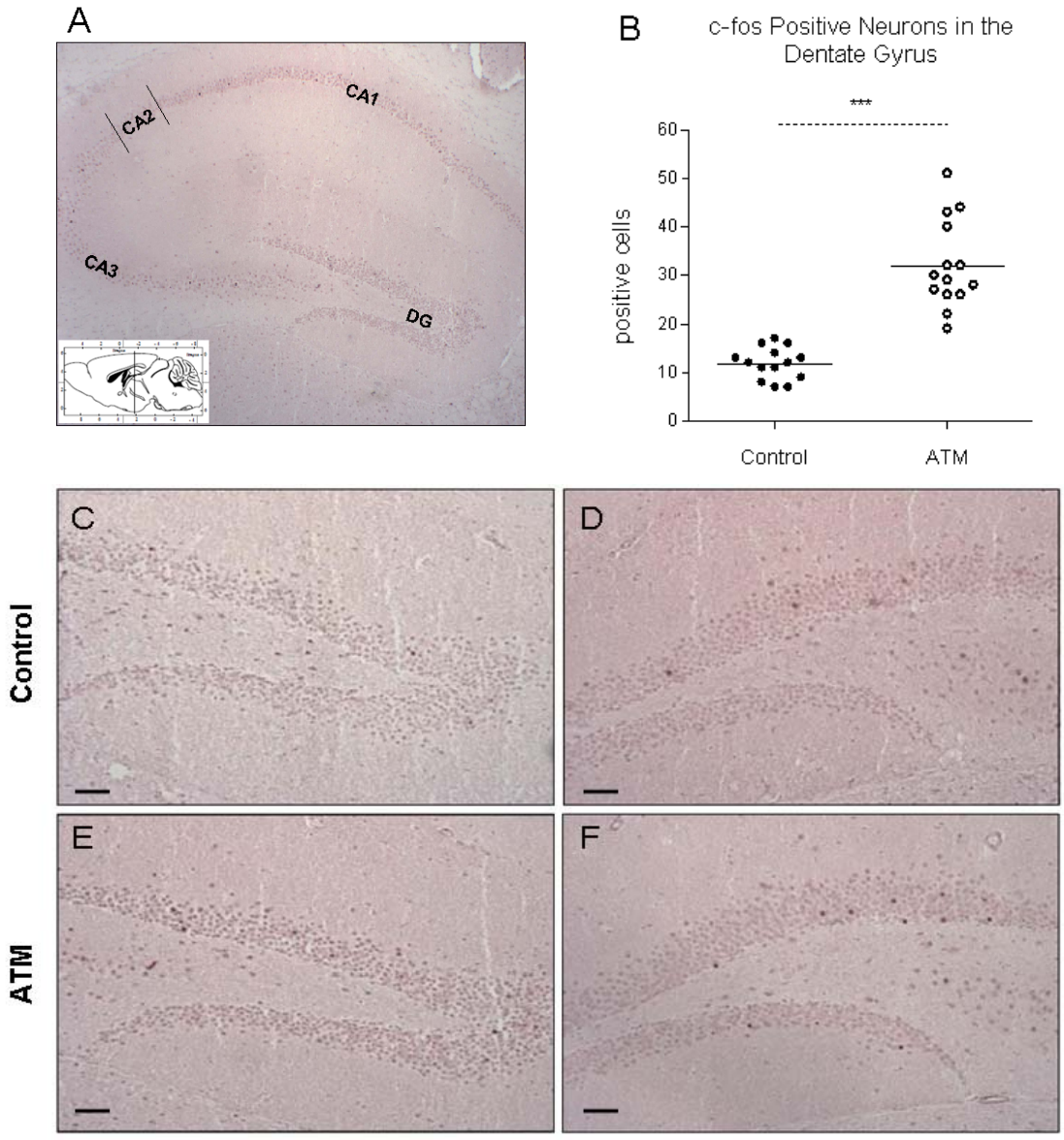
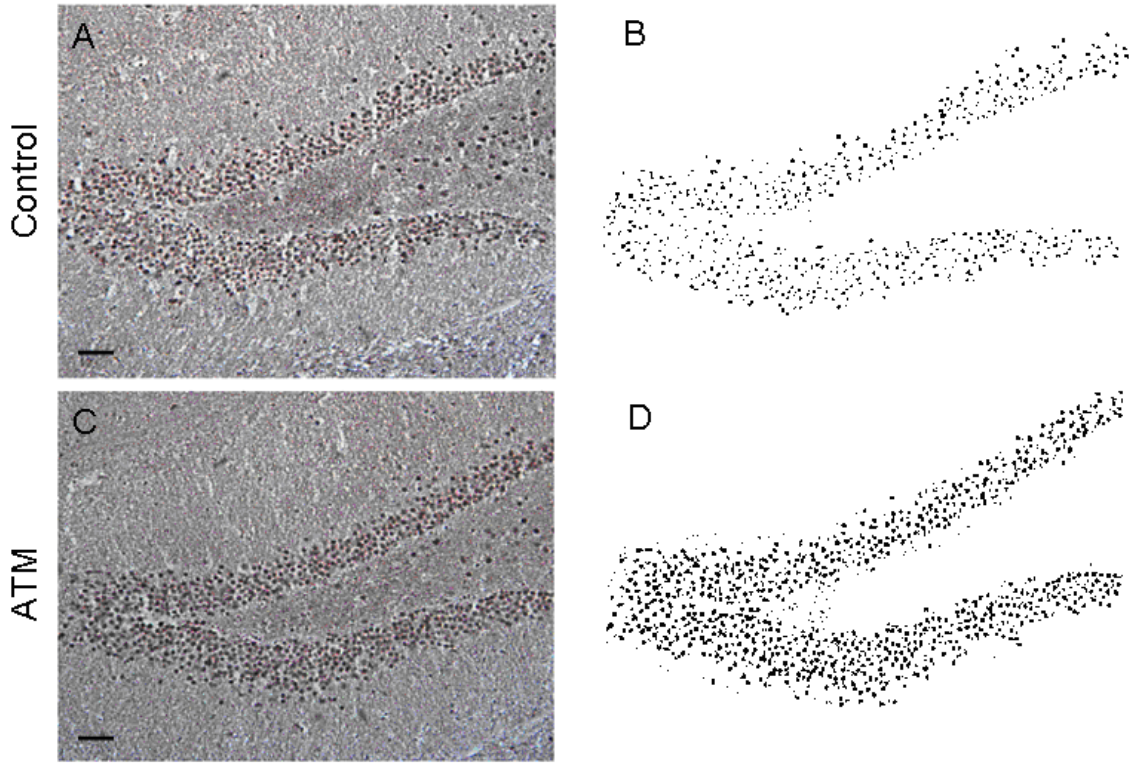


Figure 11. Perturbation of the microbiota enhances the expression of c-fos in the dentate gyrus. Coronal section using the MBSC atlas at bregma -2.06 highlighting the dentate gyrus within the hippocampal formation (A). Using ImageJ analysis software, immunopositive neurons are quantified using color thresholds, area gating and particle measurements and analyzed using a student t-test with Prism 4 statistical software (B). Sections from control and ATM-treated animals are shown (C,D and E,F respectively). Scale bars, 200μm. ***, $p<0.001$.



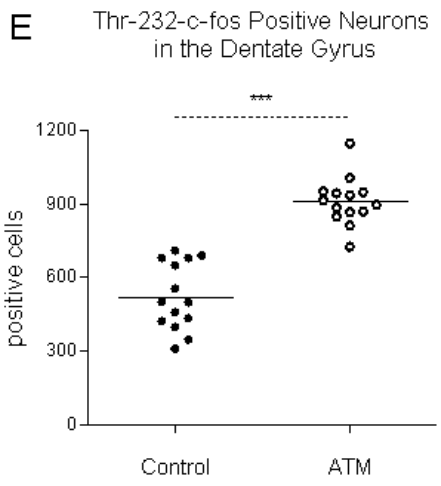


Figure 12. Perturbation of microbiota enhances the expression of Thr-232-c-fos in the dentate gyrus. Immunopositive neurons from control (A) and ATM-treated (C) animals are labelled with rabbit anti-Thr-232-c-fos antibody. Using ImageJ analysis software, immunopositive neurons are isolated and quantified using color thresholds, area gating and particle measurements (B, D). Positive cell counts are analyzed using a student t-test with Prism 4 statistical software. Scale bars, 200µm. ***, $p<0.001$.

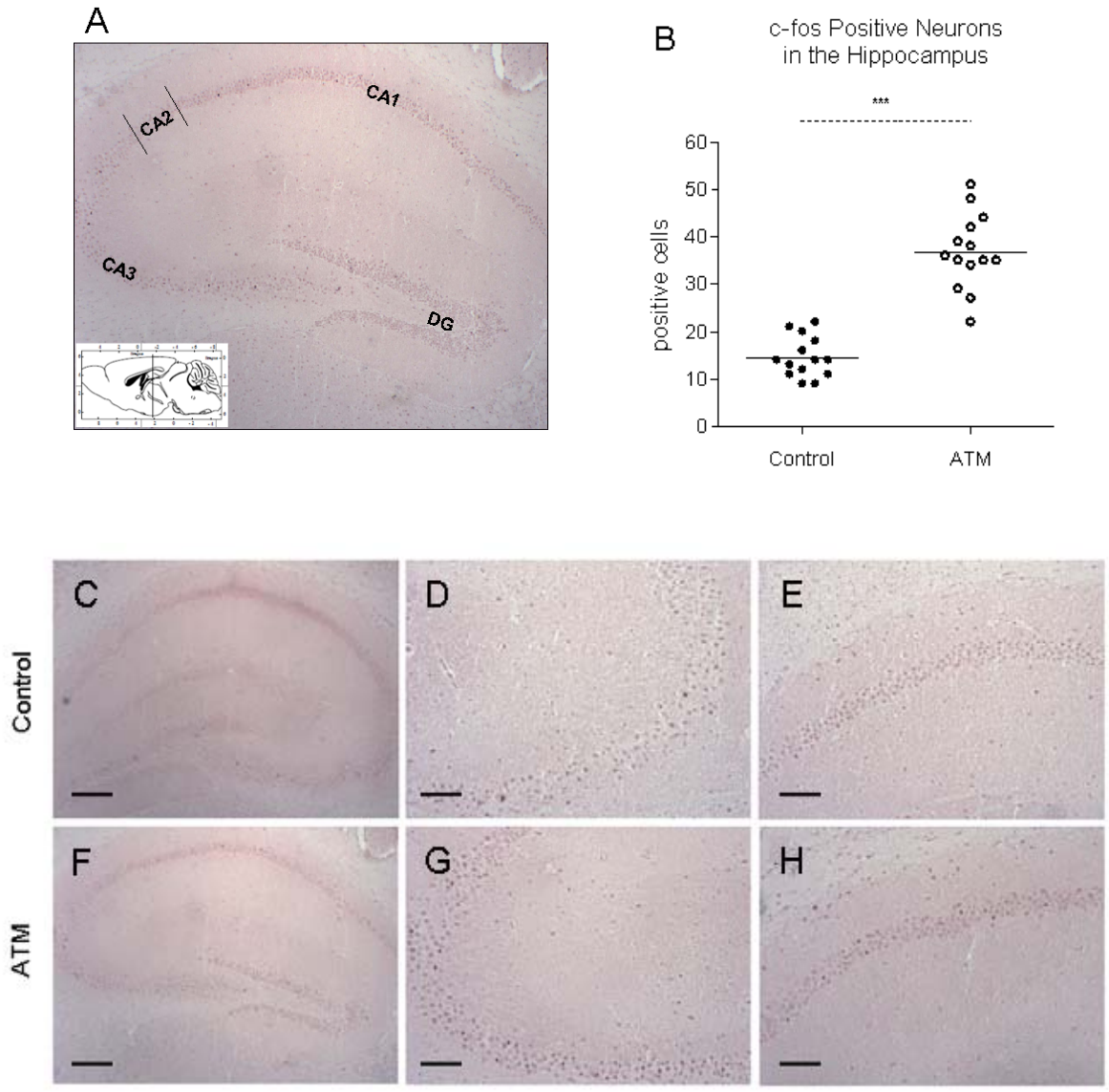
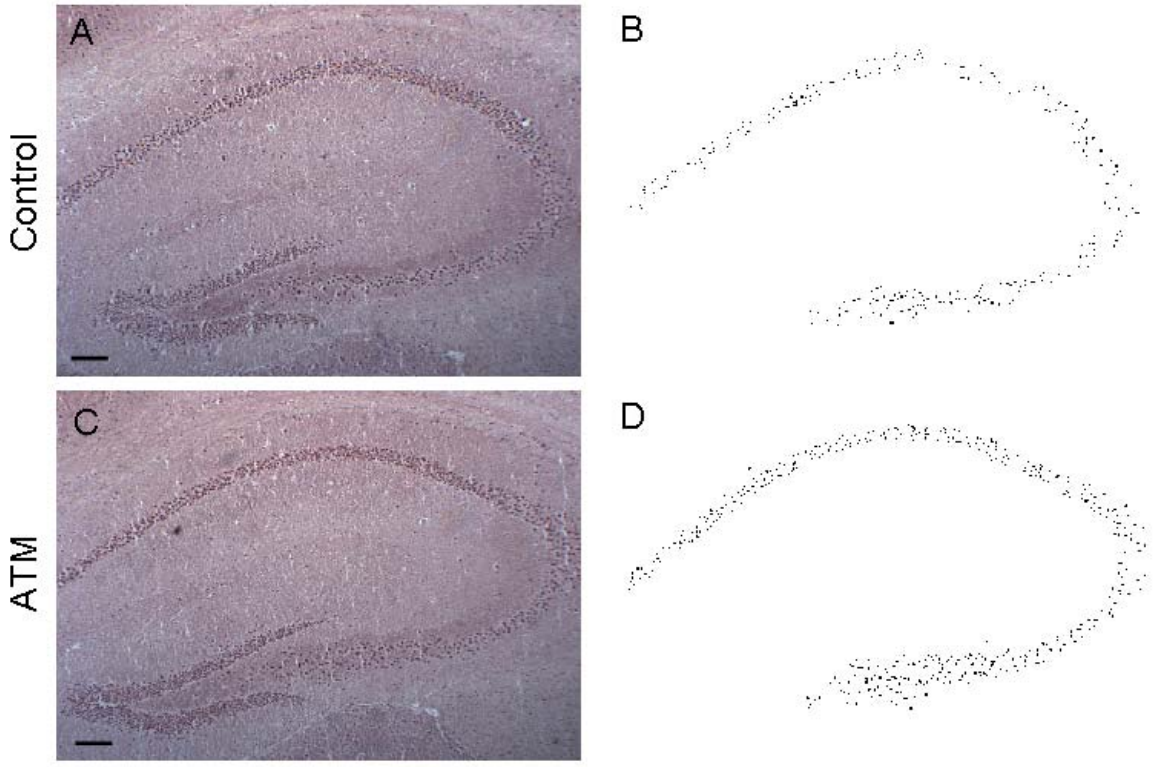


Figure 13. Perturbation of the microbiota enhances the expression of c-fos in the CA1, CA2 and CA3 regions of the hippocampus. Coronal section from the MBSC atlas at bregma -1.58 highlighting the granular layers of the hippocampus (A). Using ImageJ analysis software, immunopositive neurons are quantified using color thresholds, area gating and particle measurements and analyzed using a student t-test with Prism 4 statistical software (B). Sections from control and ATM-treated animals are shown (C,D,E and F,G,H respectively). ***, $p<0.001$.



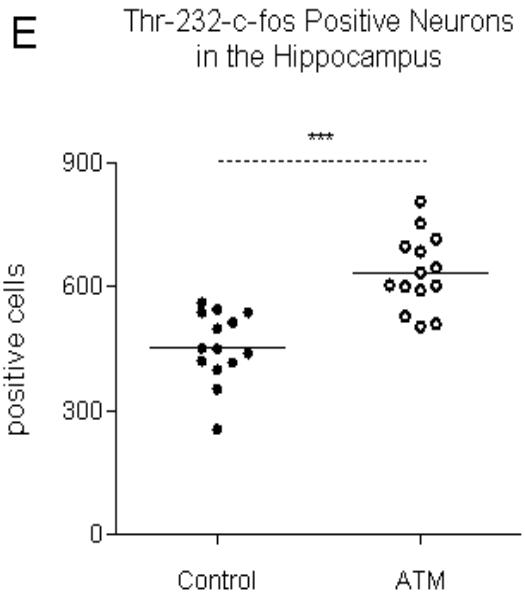


Figure 14. Perturbation of microbiota enhances the expression of Thr-232-c-fos in the CA1, CA2 and CA3 regions of the hippocampus. Immunopositive neurons from control (A) and ATM-treated (C) animals are labelled with rabbit anti-Thr-232-c-fos antibody. Using ImageJ analysis software, immunopositive neurons are isolated and quantified using color thresholds, area gating and particle measurements (B, D). Positive cell counts are analyzed using a student t-test with Prism 4 statistical software. ***,$p<0.001$.

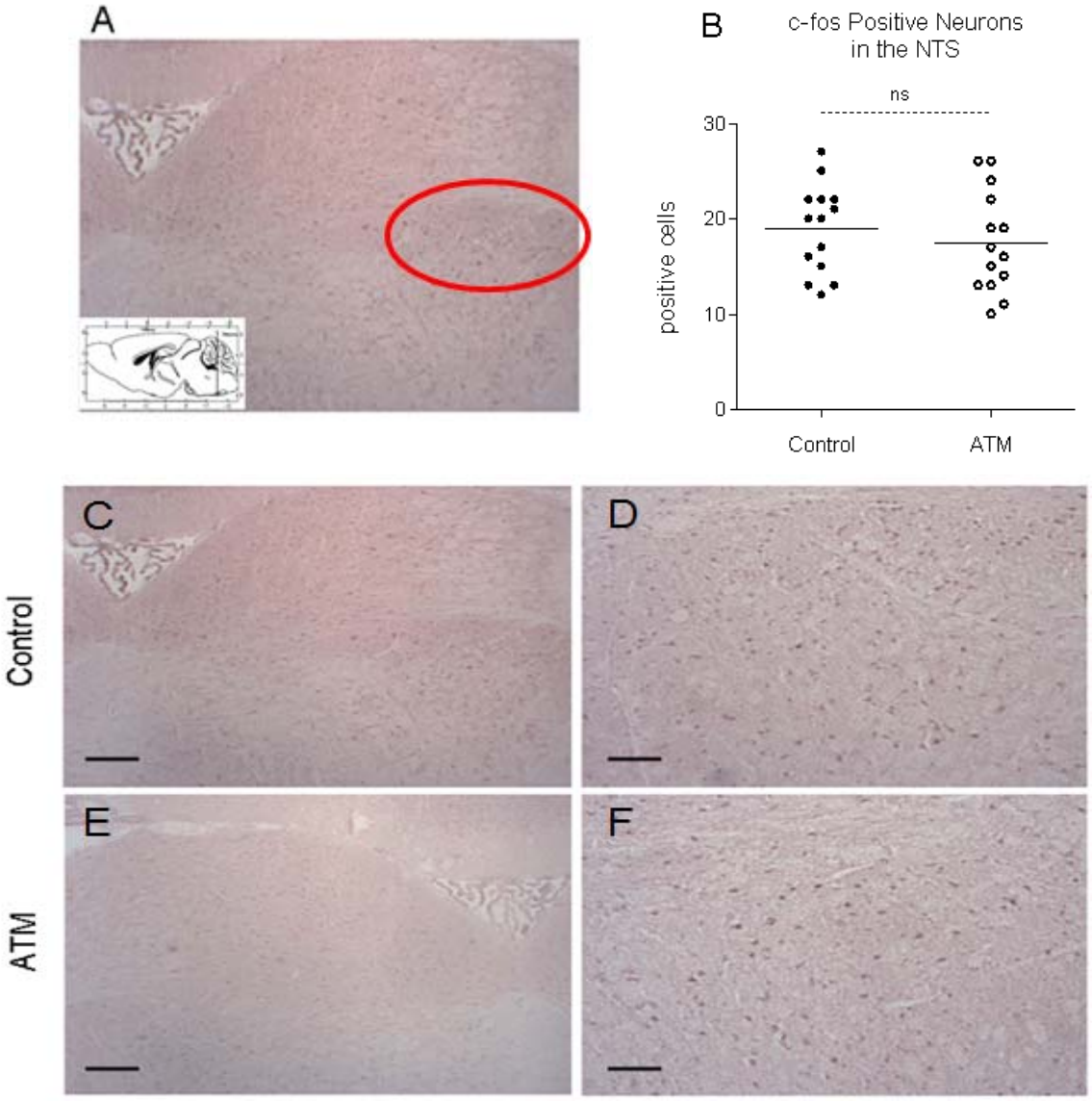


Figure 15. Perturbation of the microbiota does not influence the expression of c-fos in the NTS. Coronal section from the MBSC atlas at bregma -6.72 highlighting the nucleus of the solitary tract (A). Using ImageJ analysis software, immunopositive neurons are quantified using color thresholds, area gating and particle measurements and analyzed using a student t-test with Prism 4 statistical software (B). Sections from control and ATM-treated animals are shown (C,D and E,F respectively). Scale bars (C,E), 200µm, (D,F), 50µm. Ns, no significance.

Table 3. Mean c-fos positive neuron cell counts in respective brain regions with monoclonal c-fos and Thr-232-c-fos antibodies in control and ATM-treated mice. Data presented as means ± standard deviation, with t-test values and degrees of freedom (df).

	DG c-fos	**DG Thr-c-fos**	**Hippocampus c-fos**	**Hippocampus Thr-c-fos**	**NTS c-fos**
Control	11.86 ± 0.8762 t=7.793 df=26	521.9 ± 36.62 t=8.611 df=26	14.57 ± 1.142 t=9.276 df=26	453.5 ± 22.91 t=5.368 df=26	18.93 ± 1.247 t=0.753 df=26
ATM	32.07 ± 2.442 t=7.793 df=26	907.7 ± 25.82 t=8.611 df=26	36.79 ± 2.105 t=9.276 df=26	631.9 ± 24.08 t=5.368 df=26	17.50 ± 1.429 t=0.753 df=26

Table 4. List of Primary Antibodies

Antigen	Species	Dilution	Source
c-fos	Rabbit - monoclonal	1ug/ml	Cell Signalling Danvers, MA, USA
Thr-232-c-fos	Rabbit - polyclonal	1ug/ml	Abcam Cambridge, MA, USA
Tyrosine hydroxylase	Sheep - polyclonal	1:500	Abcam Cambridge, MA, USA

Table 5. List of Secondary Antibodies

Antibody	Conjugate	Dilution	Source
Donkey anti-rabbit	Biotin	1:200	Abcam Cambridge, MA, USA
Donkey anti-sheep	Alexa Fluor 488	1:200	Invitrogen Life Technologies Canada
Donkey anti-rabbit	Alexa Fluor 488	1:200	Invitrogen Life Technologies Canada

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