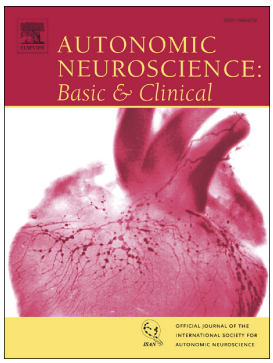

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Cholinergic Signaling Plasticity Maintains Viscerosensory Responses During *Aspiculuris tetraptera* Infection in Mice Small Intestine

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Running Title: Muscarinic Signaling in infected Mice

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Abstract

Intestinal parasites alter gastrointestinal (GI) functions like the cholinergic function. *Aspiculuris tetraptera* is a pinworm frequently observed in laboratory facilities, which infests the mice cecum and proximal colon. However, little is known about the impact of this infection on the GI sensitivity. Here, we investigated possible changes in spontaneous mesenteric nerve activity and on the mechanosensitivity function of worm-free regions of naturally infected mice with *A. tetraptera*. Infection increased the basal firing of mesenteric afferent nerves in jejunum. Our findings indicate that nicotinic but not muscarinic receptors, similarly affect spontaneous nerve firing in control and infected animals; these axons are mainly vagal. No difference between groups was observed on spontaneous activity after nicotinic receptor inhibition. However, and contrary to the control group, during infection, the muscarinic signaling was shown to be elevated during mechanosensory experiments. In conclusion, we showed for the first time that alterations induced by infection of the basal afferent activity were independent of the cholinergic function but changes in mechanosensitivity were mediated by muscarinic, but not nicotinic, receptors and specifically by high threshold nerve fibers (activated above 20 mmHg), known to play a role in nociception. These plastic changes within the muscarinic signaling would function as a compensatory mechanism to maintain a full mechanosensory response and the excitability of nociceptors during infection. These changes indicate that pinworm colonic infection can target other tissues away from the colon.

Keywords

Mechanosensitive response; Afferent multifiber recordings; Gastrointestinal sensory fibers; Acetylcholine responses; *Aspiculuris tetraptera*

1. Introduction

Pinworms from the order Oxyurida are the most common parasites in laboratory animals, with a high prevalence around the world (Pinto et al., 1994; Chen et al., 2011; Hayashimoto et al., 2013; Abdel-Gaber, 2016). In general, *Syphacia obvelata* and *Aspiculuris tetraptera* are considered as mouse pinworms, *Syphacia muris* the rat pinworm, *Syphacia mesocricetus* the hamster pinworm (Perec-Matysiak et al., 2006). The prevalence of pinworms in mice from laboratory facilities varies from 3 to 90% for *A. tetraptera* and from 9 to 90% for *S. obvelata* (Bazzano et al., 2002; Tanideh et al., 2010; Hayashimoto et al., 2013). These parasites reside in the proximal colon and the cecum of the mice, where they feed on bacteria present in the lumen (Pritchett and Johnston, 2002). Several characteristics, including the life cycle, short development period, high resistibility and easy spread of the eggs make them difficult to eliminate (Chen et al., 2011). Without proper elimination of the eggs, mice are re-exposed to the parasite by direct contact with other infected animal, contaminated food, water and bedding which results in a chronic infection that is difficult to control in the animal holdings (Michels et al., 2006).

Pinworm infected animals appear to be normal and healthy, with no obvious clinical symptoms, for this reason such infection were considered to be non or mildly pathogenic and some investigators have considered these parasites as harmless (Taffs, 1976; Pritchett and Johnston, 2002). However, others point out that they can alter experimental results and interpretation, because they can induce host humoral and cellular immune responses. Thus, mice infected with *S. obvelata* eggs show the presence of specific antibodies against the parasite somatic antigens (Sato et al., 1995), an increase of reticulocytes in peripheral blood and several white cells linages in bone marrow, and disturbed sensitivity to IL-17 (Bugarski et al., 2006). Furthermore, infection with *S. obvelata* induces Th2 immune response (IL-4, IL-5 and IL-13), a

severe phenotype in experimentally induced anaphylaxis in BALB/c mice (Michels et al., 2006), and the depression of exploratory behavior in C57BL/6 mice (McNair and Timmons, 1977).

Acetylcholine (ACh), the main neurotransmitter in the peripheral nervous system, is involved in many gastrointestinal functions through the activation of nicotinic and muscarinic receptors. Nicotinic and muscarinic receptors are present in neurons of the submucosal, myenteric (Barajas-Lopez et al., 2001; Galligan and North, 2004), and nodose ganglia (Rau et al., 2005; Hone et al., 2012). Because ACh receptors are abundant in sensory neurons, it is reasonable to expect that an altered immune response can affect the gastrointestinal afferent nerve activity. There are no reports on the impact of pinworm infection on the mesenteric nerve activity, contrary to the extensive research on the host responses elicited by other intestinal parasites, such as *Nippostrongylus brasiliensis*, *Trichuris muris*, *Trichinella spiralis* and *Schistosoma mansoni*. These parasites can alter the host mechanosensitivity (McLean et al., 1997; Keating et al., 2008), the contractility (Moreels et al., 2001; Zhao et al., 2003; Tanovic et al., 2006), ACh release (Collins et al., 1989), mucosal fluid transport mediated by nicotinic receptors (Halliez and Buret, 2015), enteric neurotransmission (De Man et al., 2001), purinergic control of cholinergic neurotransmission (De Man et al., 2003), and ACh metabolism (Davis et al., 1998; Gay et al., 2001). Because mice are often used in research and frequenly infected with pinworms, our aims in this study were to investigate if such an infection modifies the spontaneous activity and the mechanosensory response of jejunal (a worm-free region) mesenteric nerves; we also evaluated the role of the cholinergic function on these afferent activities.

2. Material and methods

2.1. Animals

Male C57BL/6 mice were obtained from the Neurobiology Institute, *Campus Juriquilla, UNAM*. Animals had access to food and water *ad libitum*. All the experiments were performed in accordance with the *Instituto Potosíno de Investigación Científica y Tecnológica* Care Committee, adhering to the official Mexican regulation (NOM-062-ZOO-1999).

2.2. Pinworm infection and identification

Two groups of mice were used; control group and mice naturally and chronically infected with pinworms. Mice infection was obtained in about 100% of the cases by housing the animals in cages containing previously infected mice during 4-6 weeks. The entire content of the large intestine and the cecum of both groups was preserved in 70% ethanol solution and then pinworms were identified by morphology (described below) and ITS. In the control group, no worms were observed by microscopic analysis, the infected group had only one genus of pinworm.

The worms were identified accordingly to their morphological characteristics and by molecular methods. We observed the specimen under a light microscope (Leica DM750) to identify principal structures and photographs were taken using a camera Leica (ICC50HD). Two pinworms (male and female) were used for scanning electron microscopy. Specimens were stored in ethanol 70%, dehydrated in a series of gradual alcohol baths, and critical-point dried and coated with a gold–palladium mixture and examined under a Hitachi SU1510 (Hitachi, Tokyo, Japan) scanning electron microscope (SEM).

For the molecular identification, two gravid female specimens were washed with saline solution and preserved in 97% ethanol. Genomic DNA was extracted individually from adult worms using a DNeasy Blood and Tissue Kit and following the manufacturer's instructions (Qiagen Inc., Valencia, CA). The internal transcribed spacer 1 (ITS1), 5.8S and internal transcribed spacer 2 (ITS2) regions of rDNA were amplified by PCR. All PCR reactions were

performed in a final volume of 25 µL (5.0 µL of 5X GoTaq® Reaction Buffer (Promega), 0.5 µL of 10 mM dNTP Mix, 3 µL of 25 mM MgCl2, 1.0 µL of each primer (10 pmol/µL), 0.12 µL GoTaq polymerase (5 units, Promega), 1-2 µL template DNA, and the remaining volume of sterilized distilled water. The ITS1, 5.8S and ITS2 regions of rDNA were amplified using the primers BD1 5'-gtc gta aca agg ttt ccg ta-3' (forward) and BD2 5'-tat gct taa att cag cgg gt-3' (reverse) (Luton et al., 1992). PCR conditions were: 5 min at 94ºC, 35 cycles of 30 s at 92ºC, 1 min 30 s at 50ºC, 1 min 30 s at 72ºC, and a final 10 min elongation period at 72ºC (modified from (Tkach et al., 2000)). PCR products were purified enzymatically with the USB® ExoSAP-IT® (Affymetrix, Inc., Santa Clara, CA), according to the manufacturer instructions, and posteriorly were sequenced (LANBAMA, IPICYT, SLP, México) in both directions on a 3500 Series Genetic Analyzer (Applied Biosystems). Chromatograms of both molecular markers were manually edited accurately using Chromas LITE 2.01 (Technelysium Pty Ltd., Brisbane, Australia). Independent searches were performed BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at NCBI and the two sequences obtained were aligned together with the sequence of *A. tetraptera* available in GenBank (EF464551.1), using ClustalW (Thompson et al., 1994), corrected manually with the computer program Bioedit v5.0.6 (Hall, 1999). Although the sequences EF464551.1 is longer than that obtained here, we used the complete segment alignment of the two sequences obtained here (1,338 pb, that include the internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 partial sequence). Uncorrected pairwise distance measures (p-distances) were calculated using the program MEGA6 (Tamura et al., 2013). Sequences obtained in this study are available in GenBank with the accession numbers KY484153 and KY484154. Voucher specimens were deposited in the "*Colección de Invertebrados no Artrópodos (CINZ) of Laboratorio de Colecciones Biológicas y Sistemática*

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2.3. Tissue preparation

Animals were sacrificed by cervical dislocation. The abdomen was opened and the intestine with the mesenteric tissue attached was excised and placed in modified Krebs solution which had the following composition (in mM): 118.4 NaCl, 24.9 $NaHCO_3$, 1.9 $CaCl_2$, 1.2 $MgSO_4$ $7H_2O$, 1.2 KH_2PO_4, 2.3 KCl, 11.7 D-glucose, that was continuously gassed with 95% O_2 y 5% CO_2. A segment of about 4 cm of jejunum was removed, washed and placed into the recording chamber that was continuously superfused with Krebs modified solution (7 mL/min) at 33 to 34°C, by gravity. The intestinal segment of jejunum was also internally perfused at a rate of 150 µL/min using a peristaltic pump with the Krebs modified solution by tying the ends up to a plastic tubes using silk thread. Afterwards, a micro-dissection of one of the mesenteric nerves was carried out. The nerve was cut in the distal part of the intestine and the nerve was secured to a glass micropipette by suction, allowing the collection of an extracellular recording of the nerve action potentials with relatively low noise.

2.4. Multiunit mesenteric nerve recordings

The micropipette was mounted to a headstage (NL 100, Digitimer Ltd) and electrical signals were amplified and filtered by an NL104 amplifier and a NL125 filter (Digitimer Ltd). Signals were digitized using an A/D converter (Micro 1401 MKII, Digitimer Ltd). The electrical activity was recorded in a computer using the Spike2 software (Cambridge Electronic Design). Once the spontaneous multiunit nerve spiking activity was obtained, the tissue was stabilized for 1 hour

before starting the experimental phase. All the drugs were applied by extraluminal perfusion with most wash intervals of 15 min and 30 min (for atropine application).

To evaluate mechanosensitivity, ramps of intraluminal pressure of jejunum segments were obtained by closing the output of intraluminal perfusion. The intraluminal pressure was continuously monitored and recorded by using a pressure transducer (NL108, Digitimer). Ramp distensions were applied at 15 min intervals to a maximal pressure of 60 mmHg. We determined the pressure–responses using a custom-made script (provided by Cambridge Electronic Design, CED) in the Spike2 interface.

2.5. Single unit analysis

Single unit discrimination by spike shape was performed offline using Spike2 waveform discriminating software. The units were classified according to their profile of response to distension (high threshold, wide-dynamic range and low threshold) as described by Booth et al. (Booth et al., 2008). This classification was based on the relative spike firing at low and high pressures (the response at 15 mmHg exhibited as a percentage of the response at 60 mmHg). Thus, low threshold spikes were those that responded >55% before 15 mmHg, high threshold spikes were those that responded <20% before 15 mmHg, and wide dynamic range spikes responded >20% but <55%, before 15 mmHg.

2.6. Semi-quantitative RT-PCR

A segment of jejunum and colon were removed from control and infested mice. The tissues were cut in small pieces by scissors and 0.1 g of tissue were homogenized with 1 ml/ Trizol® reagent (Ambion) and RNA was extracted according with the g uidelines provided by this manufacturer. RNA was quantified using a Thermo Scientific nanodrop (ND-1000) and its integrity was

verified by electrophoresis on 1.2% denaturing agarose gels. To remove DNA contamination, 5 µg of RNA were incubated during 30 min at 37ºC with Turbo DNase (2 U/µL, Ambion). DNase was inactivation with phenol/chloroform/isoamyl alcohol (Sigma) as recommended by manufacturer. Purified RNA was quantified again and its integrity was verified as described above. To synthetize cDNA we used 2 µg of RNA, 200 U of reverse transcriptase (SuperScript II, Invitrogen), and 0.5 µg of Oligo dT (Invitrogen), incubating this mix during 50 min at 42 ºC. The obtained cDNA was quantified by a Thermo Scientific nanodrop (ND-2000). Semi-quantitative PCR was performed using 750 ng of cDNA, 2.5 mM $MgCl_2$, 0.2 mM dNTps, 1 µM of each primer (see Table 1 for nucleotide sequence and Tm), and 1 U of GoTaq DNA Polymerase (Promega). Reaction conditions were as follows: initial denaturation for 5 min at 95 ºC, then 30 amplification rounds composed of denaturation for 30 s at 95 ºC, alignment for 30 s at specific temperature for each oligonucleotide (Table 1), and extension for 50 s at 72 ºC; the final extension was 5 min at 72 ºC. The PCR products were analyzed by electrophoresis in 1% agarose gel. Negative controls were performed with no cDNA template. Images were obtained with a UV Photodocumentator. Cytokine/GAPDH ratios were calculated using ImageJ application.

2.7. Histology

For some experiments, jejunum and colon segments from control and infected mice were removed, perfused intraluminally with PBS and processed for histopathological analysis, which includes: i) fixation in 4% formaldehyde, ii) embedding in paraffin (Paraffin Wax Embedding Centre, MPS/P2, SLEE Medical), iii) sectioned in 5 µm thick slices (Rotatory Microtome, CUT 6062, SLEE medical) and iv) they were stained using a Hematoxylin and Eosin standard procedure. The grade of inflammation was evaluated by a pathologist in a blinded fashion. The scoring system was as follows; No inflammation, Low-level of inflammation (infiltration of

white cells above the control animals), Intermediate-level of inflammation (macrophages and low level of epithelial damage) and High-level of inflammation (wall thickening, transmural infiltration). Image acquisition was done with an Axiocam Color (Zeiss) integrated to a ZEISS microscope (Imager M2) and using a DIC prism.

2.8. Data analysis

Multiunit afferent activity was quantified as those events with amplitudes higher than two folds the noise. Responses to the drugs were assessed by the mean change in afferent activity over a 60 s period (peak) minus the baseline frequency measured during a 100 s period. Responses to distension were measured by mean change in firing frequency every 2 mmHg subtracting the base line frequency. Multiple comparisons were made using one or two way-ANOVA with the Bonferroni *post hoc* test and the unpaired Student's *t*-test was used to evaluate differences between mean values obtained from two different groups. The data were expressed as mean ± S.E.M. and P values ≤ 0.05 were considered statistically significant.

2.9. Drugs

All drugs were purchased from Sigma-Aldrich. The stock solutions (1 M) of hexamethonium was prepared with water as a solvent and in DMSO for atropine (100 mM).

3. Results

3.1. Morphological and molecular identification of pinworms

The nematodes were identified as members of the family Heteroxynematidae, the genus *Aspiculuris*. This nematode presents a sexual dimorphism (male shorter than female), possesses a

medium size and cylindrical body, is characterized by distinctive cervical alae, which begin immediately posterior to the cephalic vesicle and is abruptly interrupted at level of esophageal bulb, forming an acute angle (Fig 1). The anterior end of the body has a prominent inflated region, which represents the cephalic vesicle. Males have 3 pairs of alae in the tail (Falcón-Ordaz et al., 2010; Liu et al., 2012).

Using molecular evidence based on the internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, partial sequence regions of rDNA, we corroborated that mice were infected by the specie *Aspiculuris tetraptera*. The alignment was 1,338 pb in length without insertions, two sequences obtained here were identical and 99.9% of similarity to the *A. tetraptera* sequence (EF464551.1) available in GenBank (Table 2). The regions analyzed are highly conserved and provide sufficient information at this taxonomic level to confirm the molecular identification. There is only one change in EF464551.1 (G to A in our sequences), located at the position 836 in the internal transcribed spacer 2 regions (Table 2).

3.2. Higher spontaneous mesenteric nerve activity was found in A. tetraptera infected mice

We observed a significantly higher spontaneous nerve firing in the infected group than in the control group, average values were 50±3 and 34±2.7 spikes/s, respectively (Fig 2A). The increase in spontaneous firing activity in the infected group is likely independent of changes in cholinergic function because it was decreased in a similar proportion by hexamethonium and atropine did not induce any further decrease in any of the two groups (Fig 3). This indicates that there is a tonic release of ACh that modulates spontaneous afferent nerves activity of mouse jejunal segments in control and infected groups by activating mainly nicotinic receptors.

3.3. Role of cholinergic receptors in mechanosensitivity responses

Firing activity increased when ramps of intraluminal jejunal distension were applied. This increase was typically biphasic and this pattern response was not different between both mice groups (Fig 2B). ACh is a major neurotransmitter in the mammalian gut and plays an important role in modulating gastrointestinal functions, including during inflammatory conditions (Galligan and North, 2004). Therefore, we investigated the role of cholinergic signaling in control and infected animals. We measured firing activity before and in the presence of a maximal concentration of hexamethonium (a blocker of nicotinic receptors; 1 mM) alone or together with atropine (1 μM), a potent muscarinic receptor antagonist that also blocks nicotinic receptors (Barajas-Lopez et al., 2001).

It was found that the cholinergic antagonists did not modify spiking activity induced by distension in the control group (Fig 4A), but atropine plus hexamethonium inhibited it significantly in infected mice (Fig 4B). Such an effect was exclusively observed with gut pressures above 20 mmHg. No significant differences were seen in the nerve response to distension by using hexamethonium alone in infected mice. This indicates that muscarinic receptors are involved in the response to intraluminal distension in the infected but not in control mice.

3.4. Cholinergic inhibitors decrease the high threshold spike responses in infected mice

Single unit analysis of afferent responses to intraluminal distensions was carried out using Spike2 waveform discriminating software. Single units were classified based on the spike morphology. This allowed us to examine the effect of various experimental variables on individual fibers. Fig 5 shows three different response profiles of individual fibers from jejunal segments from a

control and an infected mice; the low threshold spikes responded with a fast increase of nerve activity at low pressures, the wide dynamic range spikes responded slower with an increase directly proportional to the pressure, the high threshold spikes responded at pressures up to 15 mmHg (Booth et al., 2008). We also found an increase in relative spontaneous single unit frequency of the infected animals (Table 3), which correlates well with the increase in spontaneous spiking frequency in the whole mesenteric nerve (see above).

In control mice, cholinergic receptor antagonists (1 mM hexamethonium alone or together with 1 μM atropine) did not change the activity during ramps of distension of any of the three fiber types (Fig 6A1-A3). However, in infected mice, hexamethonium (1 mM) plus atropine (1 μM), decreased significantly the response to distension in the high threshold units (Fig 6B3). Hexamethonium by itself did not affect any unitary activity. These observations would indicate that muscarinic receptors are, at least in part, responsible for the activation of high threshold fibers during distension but only in the infected group.

3.5. Proinflamatory cytokines were elevated by infeccion but not changes were noticed in histological analysis.

Chronic infections with helminths have been associated with activation of an innate immune response (Moncada et al., 2003). However, *T. spiralis*, *S. mansoni*, and *N. brasiliensis* can also induce a Th1 response, mainly causing damage to the epithelial cells. Opposite to these findings, *S. obvelata* does not induce inflammation, goblet hyperplasia or goblet response even in the colonized colon (Marillier et al., 2008). In the present study we analyzed histological sections of control and *A. tetraptera* infected mice (Fig 7) and we did not notice any cytological difference in sections coming from either group, compatible with inflammatory damage in infected mice. We also measured the expression of mRNAs, using a semiquantitative analysis, of three known

inflammatory cytokines (IL-1ß, IL-6, and IFN-γ) in both groups of mice. We found that IFN-γmRNA levels were increased in the jejunum and colon of infected versus the control animals (Fig 8). IL-6 increased only in the colon of infected versus de control animals.

4. Discussion(Messenger and Furness, 1993)

Observations from this study, extend our understanding on how the afferent neuronal signaling is modified by gut infection with *A. tetraptera* in mice. Chronic infection with this worm increases both, the intestinal spontaneous firing and the participation of muscarinic receptors in the distension response for high threshold fibers. These changes in mesenteric nerve activity are seen in the jejunum, a worm-free segment, while this *A. tetraptera* is known to colonize the colon.

Analogous to what it was found here, previous reports have documented alterations in gastrointestinal functions in worm-free regions in animals infected with *T. spiralis*; hypercontractility induced by carbachol (an agonist of ACh receptors) on intestinal worm-free regions (Marzio et al., 1990), similar results were obtained in a more recent study (Tanovic et al., 2006), who observed higher contractile responses to KCl and ACh in inflamed and non-inflamed intestinal areas in rats. Such changes occur in the absence of any apparent inflammatory cell infiltrate in the mucosa or myenteric plexus, however, it is known that initiation of the hypercontractility state requires CD4 cells and Th2 cytokines acting via STAT6 pathways (Mayer and Collins, 2002), which would indicate that impaired function in non-inflamed area is the result of a generalized response to the infection. Thus, the increase in spontaneous activity and the larger involvement of muscarinic receptors in the distension response, observed here, is likely the result of a generalized response of the host to *A. tetraptera* interaction in the colon. In support of this hypothesis, mice infected with *S. obvelata* exhibit a Th2 immune response (IL-4, IL-5 and IL-13), an elevation of blood reticulocytes, alterations in various white cells linages in the bone marrow and in the sensitivity to IL-17 (Mayer and Collins, 2002), a severe phenotype in experimentally induced anaphylaxis (Michels et al., 2006), and depression exploratory behavior (McNair and Timmons, 1977).

As with *S. obvelata,* other parasites, like *T. spiralis*, *S. mansoni*, and *N. brasiliensis,* can induce a Th2 immune response, characterized by an elevated IL-5, IL-4 and IL-13. These last two interleukins, have been shown to increase external muscularis contractility and to decrease ACh release (Akiho et al., 2002; Zhao et al., 2003; Hewitson et al., 2009). Chronic infections with helminths are associated with activation of an innate immune response and such regulation has a crucial role in parasite expulsion (Moncada et al., 2003). However, *T. spiralis*, *S. mansoni*, and *N. brasiliensis* can also induce a Th1 response, mainly causing damage to the epithelial cells. Nevertheless, *S. obvelata* does not induce inflammation, goblet hyperplasia or goblet response even in the colonized colon (Marillier et al., 2008), as it was also found here during infection with *A. tetraptera*. Nevertheless, it was noticed that the mRNA levels of at least some pro-inflammatory cytokines (IFN-γ and IL-6) were higher in both the jejunum and colon of infected animals, which suggests that a marginal inflammatory response is present at some point of the *A. tetraptera* infeccion. Previous studies have found that these two cytokines can target and alter the nervous system (Zhang and An, 2007; Garcia-Oscos et al., 2015; Sonekatsu et al., 2016). We proposed additional analysis of this hypothesis using real time PCR at different times of the infeccion, which is clearly out of the scope of the present study.

In the present study, we found an increase in the mesenteric spontaneous spiking activity in mice with a chronic infection of *A. tetraptera*. Similar findings have been reported 1 to 2 months after infecting mice with *T. spiralis*, when the inflammatory response has subsided (Keating et al., 2008). In line with such findings, hyperexcitability was reported in neurons of the dorsal root ganglia, nodose ganglia, and myenteric plexus of mice infected with *T. spiralis* and *N. brasiliensis* (Aerssens et al., 2007; Keating et al., 2008; Rong et al., 2009). Also, in other post-infection models, like *C. rodentium*, an increase in afferent spontaneous firing and

hyperexcitability of dorsal root ganglia neurons has been demonstrated (Ibeakanma et al., 2009; Ibeakanma et al., 2011), despite the fact that the host immune response induced by this bacterium is different than that induced by intestinal worms (Patel et al., 2009).

A. tetraptera infection did not change the whole afferent nerve activity induced by distension compared with the control group (present study), which agrees with the lack of effects in intestinal afferent mechanosensitivity observed in the jejunum of BALB/c mice infected with *N. brasiliensis* (Aerssens et al., 2007). In contrast, other studies have reported an increase in the mechanical sensitivity. Thus, it has been noticed an increase in systemic blood pressure induced by jejunum distension in rats infected with *N. brasiliensis* (McLean et al., 1997) and increment in afferent mesenteric response to ramp distensions in mice at 4 to 8 weeks post-infection with *T. spiralis* (Keating et al., 2008). In addition, it has being reported an increase in visceral sensitivity (evaluated by abdominal electromyography) in rats infected with *T. spiralis* elicited by colorectal distension (Yang et al., 2009)

For the first time, we found that muscarinic receptors were responsible, at least in part, for the nerve response to distension in the infected group but no in the controls. Our data show that high threshold fibers are the component mediating such changes. In previous studies, these fibers have been correlated with gut nociceptors (Grundy, 2006). Nicotinic receptors do not appear to be involved because hexamethonium alone fails to change the response to distension. Nerve spiking response to bladder distension, in control mice, appears to be modulated by muscarinic receptors differently (Daly et al., 2010) than the jejunum (present study). Thus, in the bladder, muscarinic receptor agonists decrease the mechanosensory nerve response whereas in the jejunum, endogenous activation of muscarinic receptors is facilitating this response in the infected mice whereas, these receptors appear not to be involved in the control group. However, analogous to those findings reported here, atropine failed to affect the response to distension in

the bladder of control mice. Clearly, further research is needed to identify the mechanisms and type of muscarinic receptors that fine-tune the response to endogenous ACh release in the infected mice, which targets high threshold fibers specifically. We hypothesize, that one mechanism is through the modulation of transient receptor potential cation channels (TRPs), which are widely modulated by muscarinic receptors (Woo et al., 2008; Adapala et al., 2011; Bavencoffe et al., 2011; Birrell et al., 2014). In support of this hypothesis, transcripts encoding for various muscarinic receptors are found in dorsal root ganglia neurons (Tata et al., 2000) including those expressing TRPV1 (Woo et al., 2008). However, this and other possible mechanisms, require to be investigated experimentally.

The fact that nicotinic receptors, but not muscarinic, modulate part of the spontaneous nerve activity and that muscarinic receptors, but not nicotinic, modulate part of the High threshold fibers suggests that these two cholinergic receptors are located at distinct nerve populations. This interpretation is further supported by studies showing that spontaneous nerve activity is mainly dependent on firing of vagal afferents (Booth et al., 2008), which are in part activated by serotonin through the activation of 5-HT_3 receptors and appears not to be mechanosensitive (Aerssens et al., 2007); whereas, High threshold fibers are mainly spinal afferents, related with nociception (Grundy, 2006). Myenteric neurons have also been reported to project to sympathetic prevertebral ganglia but this appears to be mainly from the colon (Messenger and Furness, 1993; Miller and Szurszewski, 1997), and most likely contribute little to the mesenteric activity recorded here.

In summary and for the first time, we have demonstrated in mice that spontaneous gut infection with *A. tetraptera*, alters the basal mesenteric nerve activity but not the mechanosensory response in the jejunum, a worm free gastrointestinal segment. Spontaneous nerve activity, increased by infection, was independent of cholinergic function. No change was observed in the

full mechanosensory response to jejunal distension by infection but part of this response was dependent on muscarinic receptor activation revealing a higher cholinergic tone in the jejunum of these animals. Furthermore, our findings indicate that nicotinic but not muscarinic receptors similarly affect spontaneous nerve firing in control and infected animals; these axons are believed to be mainly vagal. Whereas, only in the infected group, muscarinic but not nicotinic receptors modulated specifically high threshold nerve fibers, which are thought to be spinal afferents and to play a role in nociception. One role for this higher cholinergic tone would be to maintain the full mechanosensory response and the excitability of nociceptors during colonic infection.

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Tables

Table 1. Oligonucleotides used in RT-PCR of mice

Transcript of interest	*Oligonucleotide Sequence*	*Tm (ºC)*
GAPDH	Fw 5'-GTCATCATCTCCGCCCCTTC -3' Rv 5'-CATTGAGAGCAATGCCAGCC -3'	65
INFγ	Fw 5'-CCACGGCACAGTCATTGAAA -3' Rv 5'-TCTCTTCCCCACCCCGAAT -3'	56
IL-1β	Fw 5'-TGCCACCTTTTGACAGTGATG -3' Rv 5'-TGGGTGTGCCGTCTTTCATT -3'	60
IL-6	Fw 5'-TCCGGAGAGGAGACTTCACA -3' Rv 5'-TAACGCACTAGGTTTGCCGA -3'	60

Table 2. Alignment of the two sequence with *A. tetraptera* (EF464551.1) based on the internal transcribed spacer 1 (1 to 662 pb), 5.8S ribosomal RNA gene (663 to 822 pb), and internal transcribed spacer 2 partial sequence (823 to 1338 pb). Arrow indicates the nucleotide difference observed here and dots indicate identical nucleotides.

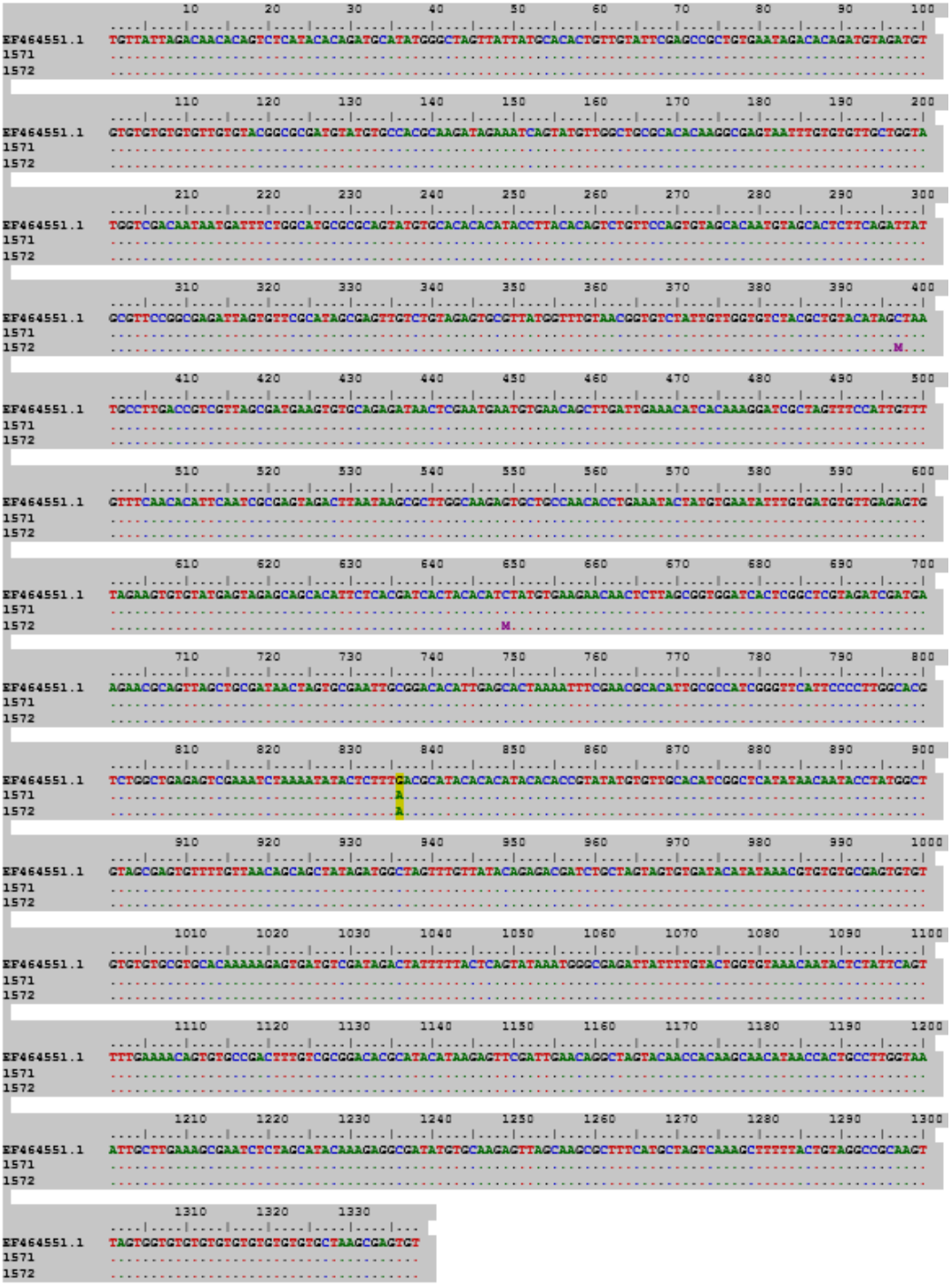

Table 3. Spontaneous multiunit and unitary spiking activity in control and infected mice

	Intestinal segments (n)	Spikes/s	P value
Multiunit activity			
CONTROL	24	34.4±2.7	<0.001
INFECTED	23	50.3±3.0	
Single unit activity			
CONTROL	10	4.2±0.4	<0.03
INFECTED	8	5.9±0.4	

Figures legends

Figure 1. Characteristic structures of the genus *A. tetraptera*. **A)** Anterior end as observed with an optical microscope. Blue arrow shows the Cephalic vesicle. Black arrow indicates the cervical alae which begins immediately posterior to the cephalic vesicle and is abruptly interrupted at level of the esophageal bulb, forming an acute angle. **B)** Anterior end of the body by SEM. **C)** Mouth apical view, with three lips. **D)** Ventral view of male, lateral alae in both sides (black arrow). **E)** Vulva region of female, lateral view. **F)** Egg, ovoid and symmetrical. **G)** Male tail, lateral view. Black arrow points the Pre-cloacal alae. Blue arrow points the pre-cloacal papillae. **H)** Posterior view of male, caudal papillae arrangement. Blue arrow points the post-cloacal papillae. Black arrow points the ad-cloacal papillae. Green arrow points the pre-cloacal papillae

Figure 2. Spontaneous nerve firing activity is increased in infected mice. A) Spontaneous nerve activity from mice jejunal segments of control and infected groups. Spontaneous activity was significantly higher in the infected versus the control group ($P<0.001$, control n=24, infected n=23), bars represent the mean ± S. E. M. **B)** Change in nerve activity as a function of the intraluminal pressure in control and infected groups, each point represents the mean±S.E.M. Nerve activity increased as the intraluminal pressure augmented in a similar manner. There were no significant differences between control (n=10) and infected (n=8) groups.

Figure 3. A low proportion of Spontaneous nerve firing activity involves Nicotinic but not muscarinic receptors in both groups. The frequency of spikes decreased when hexamethonium (1 mM) was added but no additional effect was observed when atropine (1 μM) was superfused (control n=10, infected n=13). Bars represent the mean ± S. E. M. (*, $P<0.05$).

Figure 4. The response to distension involved muscarinic receptors but only in the infected group. **A**) Nerve activity as a function of the intraluminal pressure in control mice (n=5). Before, and in the presence of hexamethonium or hexamethonium plus atropine, was virtually the same.

B) In the infected group (n=5), did not significantly decreased nerve activity; however, nerve responses were significantly lowered by addition of hexamethonium plus, but only above 20 mmHg (*, P<0.05)

Figure 5. Spikes can be sorted by their morphology and intraluminal pressure sensitivity. Top panel shows the increase in intraluminal pressure when a ramp distension protocol was applied to the jejunal segments. Second panel (RAW) shows all spikes sorted from a representative experiment in a control (**A)** and an infected (**B)** mouse. Each color represents a different waveform sorted with the Spike 2 software. Third panel (TOTAL) is a histogram frequency of all these spikes. Next lower panels show frequency histograms of high threshold (HIGH), wide dynamic range (WDR), and low threshold (LOW) spikes. High threshold spikes were classified as those with a response lower than 20% (of the response observed between 50 to 60 mmHg) before 15 mmHg. Wide Dynamic Range spikes were taken as those responding to widespread pressures with more than 20%, but lower than 55%, before 15 mmHg. Low threshold spikes were those that responded more than 55% before 15 mmHg.

Figure 6. In infected mice, muscarinic receptors are involved in the response of High threshold spikes to jejunal distension. The graphs show the profile of the afferent nerve responses to distension after the sorting of the spikes by waveform. **A)** Afferent activity in response to distension in control mice (n=5): **A1)** Low threshold, **A2)** Wide Dynamic Range and **A3)** High threshold fibers. **B)** Afferent activity in response to distension in infected mice (n=5): **B1)** Low threshold, **B2)** Wide Dynamic Range and **B3**) High threshold fibers. Each point represents the mean ± S. E. M. The response of High threshold fibers to distension in the presence of hexamethonium plus atropine decreased significantly (*, P<0.05).

Figure 7. **No differences were found in the Jejunum and Colon inflammation scores.** Microphotographs of jejunum and colon slices from control (on the left) and infected (on the

right) mice, stained with hemathoxilin & eosine. Tissues from infected animals are similar to those from control group.

Figure 8. **Expression of proinflammatory cytokines increase in the jejunum and colon of infected mice.** Semi-quantitative RT-PCR of mRNA extracted from jejunum (**A**) or colon (**B**) of control and infected mice. Cytokine mRNA expression was quantified by densitometry analysis of the gels. This relative expression of cytokine transcripts is shown as a fraction of GAPDH expression (n=4 for each group). Bars and lines on their top represent the mean ± S. E. M. (*, $P<0.05$), respectively.

FIGURE 1

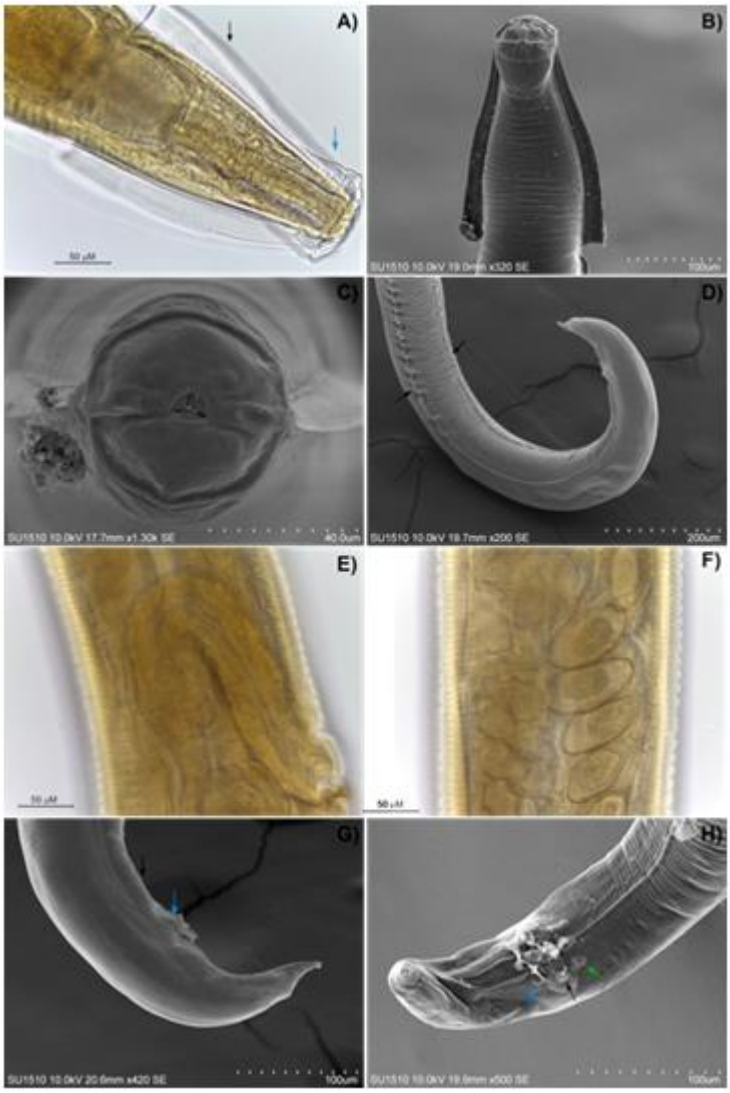

FIGURE 2

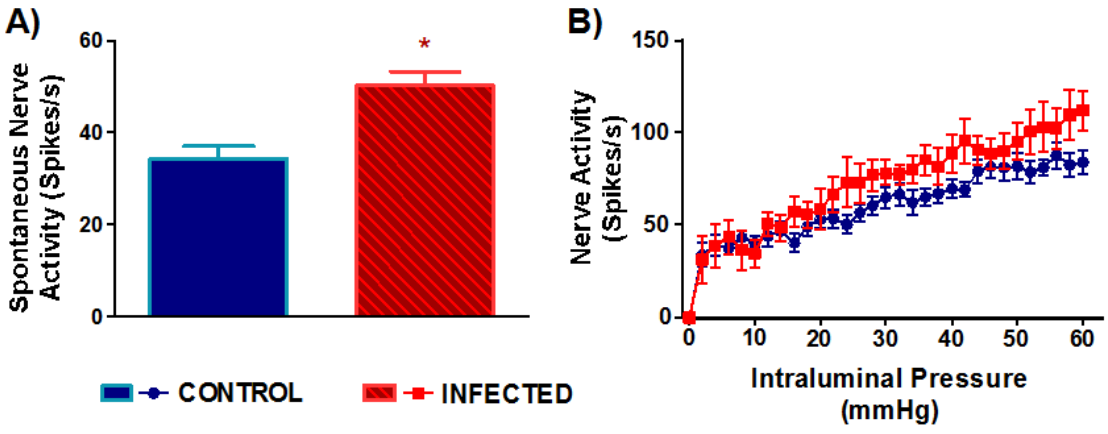

FIGURE 3

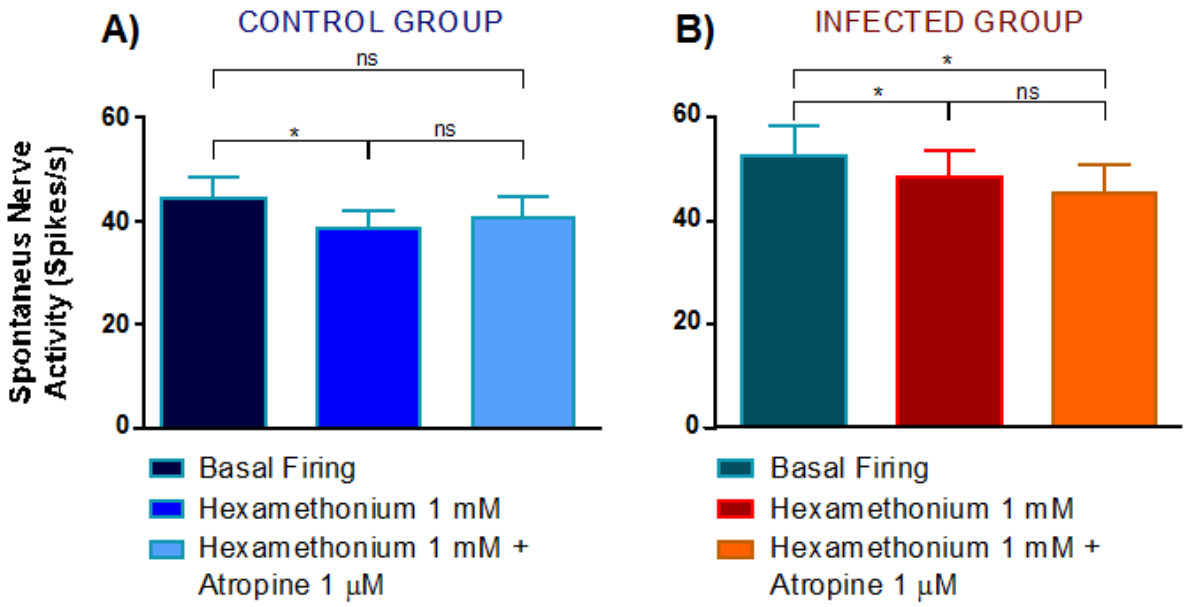

FIGURE 4

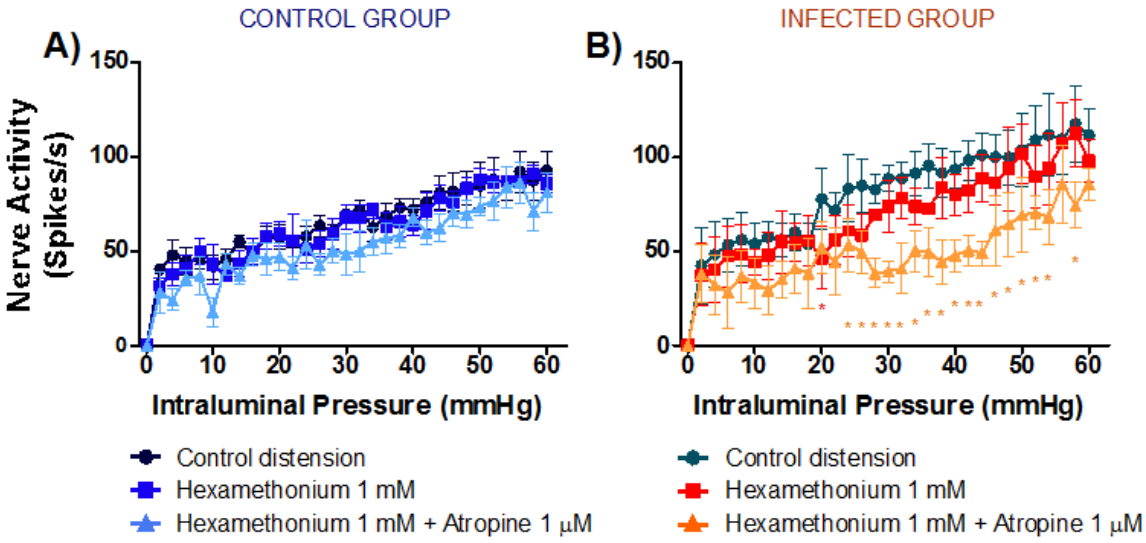

FIGURE 5

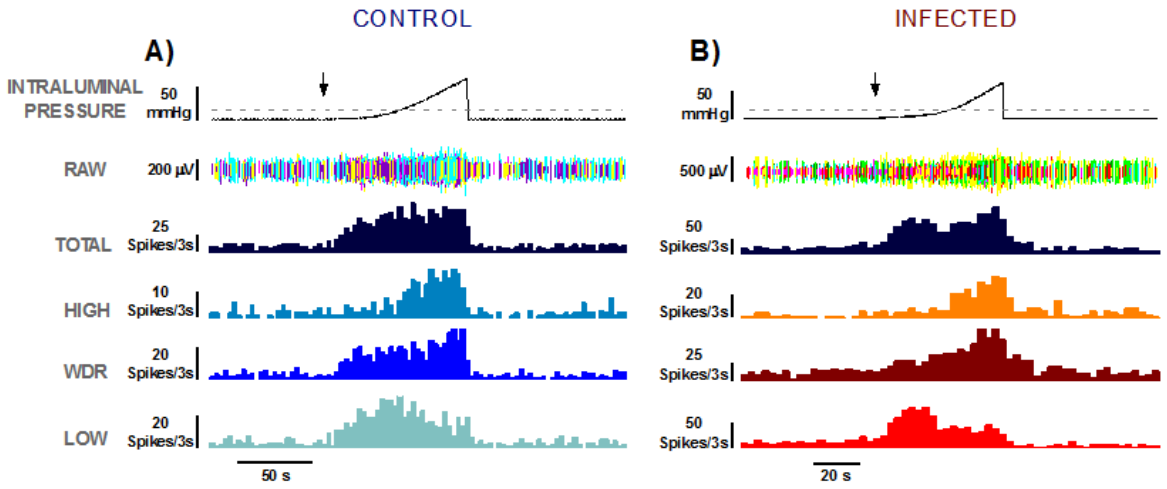

FIGURE 6

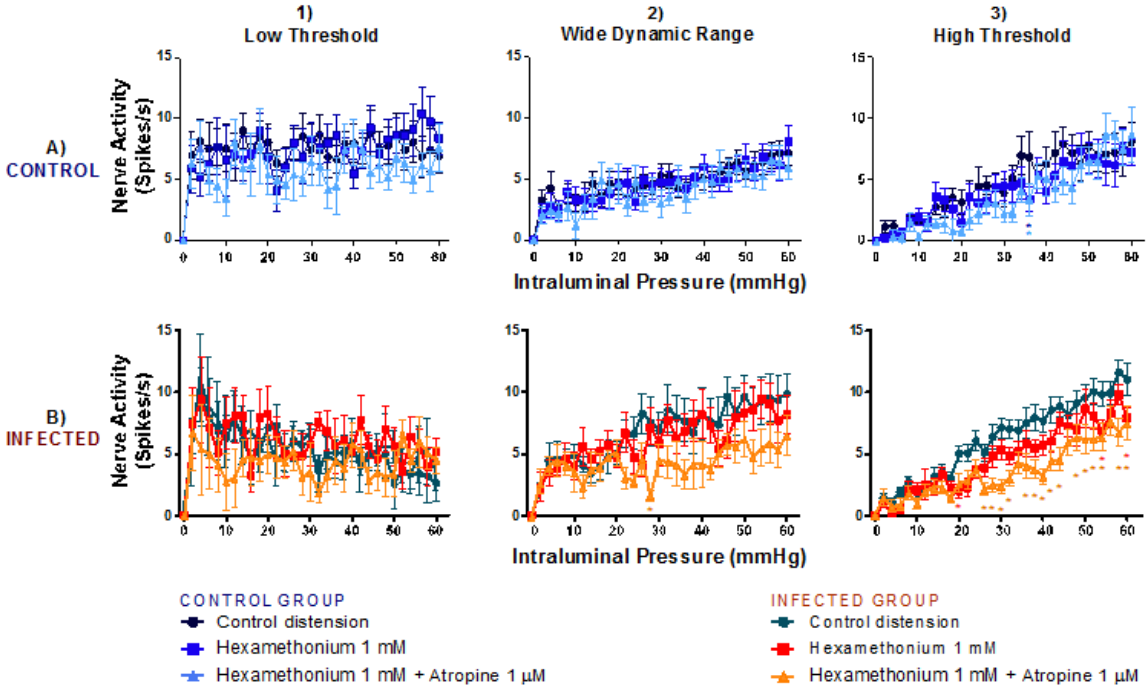

FIGURE 7

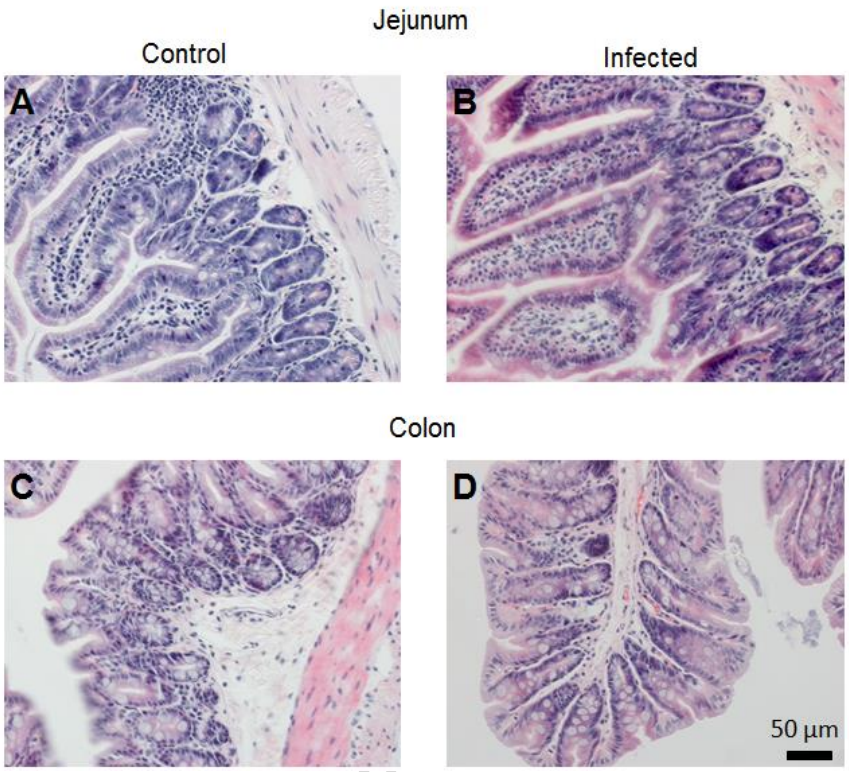

Figure 8