INFLUENCE OF MICROBIAL PRODUCTS ON THE DEVELOPMENTAL PROGRAMMING OF THE ENTERIC NERVOUS SYSTEM

INFLUENCE OF MICROBIAL PRODUCTS ON THE DEVELOPMENTAL PROGRAMMING OF THE ENTERIC NERVOUS SYSTEM

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ABSTRACT

Bacterial colonization of the gastrointestinal (GI) tract takes place during the perinatal period, thus coinciding with a critical window of enteric nervous system (ENS) development. Previous work has found that the myenteric plexus of germ free (GF) mice exhibits structural and functional aberrancies in the early postnatal period as compared to specific pathogen free (SPF) and altered Schaedler flora (ASF) mice. These early life disruptions in ENS development in GF mice compared to SPF mice, and more specifically ASF mice, support the notion that a simple intestinal flora is sufficient for directing perinatal ENS development.

It has previously been believed that the intrauterine environment during fetal development is sterile. Recent evidence showing successful isolation of microbial communities from embryonic cord blood and newborn meconium that are not of maternal origin suggests that the intrauterine environment is not sterile and is unique to the fetus. Coinciding with this timeline of fetal microbial colonization is the development of the ENS through a population of precursors known as enteric neural crest derived cells (ENCDCs). The prenatal period is characterized by rapid expansion and differentiation of ENCDCs into the many enteric neuron subtypes that comprise the ENS. Terminal differentiation of ENCDCs continues into the early postnatal period.

In the current study, we tested the hypothesis that ENCDCs interact directly with microbial products during ENS development. Further, these ENCDC-bacterial product interactions influence the proliferation, apoptosis, and chemical coding of enteric neuron precursors. These objectives were carried out in an *in vitro* model of ENCDCs isolated

from the prenatal period that was established for the first time in our lab using immunoselection. Further, this model was characterized at key timepoints for proliferation, apoptosis, and differentiation.

Our results are suggestive of direct ENCDC interactions with lipopolysaccharide (LPS), a TLR4 ligand, and flagellin, a TLR5 ligand, in stimulating ENCDC proliferation and differentiation into early born neurons of nitrergic and serotonergic subtypes. Peptidoglycan derivatives, muramyl dipeptide (MDP) and ɣ-D-Glu-mDAP (iE-DAP), ligands for NOD2 and NOD1 respectively, appear to mainly stimulate differentiation into nitrergic neurons, and possibly serotonergic neurons. The lack of apoptosis in all conditions is consistent with the notion that apoptosis is not an important characteristic of ENCDC maturation and ENS development. Finally, the lack of significance for differentiation into dopaminergic neurons could be further evidence of their late born nature, which has previously been reported to be stimulated by serotonin after the emergence of serotonergic neurons.

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5-HT	5-hydroxytryptamine
7-AAD	7-aminoactinomycin D
BDNF	brain-derived neurotrophic factor
bFGF	fibroblast growth factor
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CCL20	C-C motif chemokine ligand 20
CGRP	calcitonin gene-related peptide
CXCL1/3	C-X-C motif chemokine ligand 1/3
DMEM	dulbecco's modified eagle media
DMSO	dimethyl sulfoxide
EDNRB	endothelin receptor type B
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2´-deoxyuridine
EGF	epidermal growth factor
ENCDC	enteric neural crest derived cell
ENS	enteric nervous system

ET3	endothelin 3
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
GDNF	glial cell line-derived neurotrophic factor
GF	germ free
GI	gastrointestinal
HEK cells	human embryonic kidney cells 293
HPA	hypothalamic-pituitary axis
IBD	inflammatory bowel disease
iE-DAP	gamma-D-Glu-mDAP
IgG	immunoglobulin G
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
MDP	muramyl dipeptide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK4/IL-32	interleukin 32
nNOS	nitric oxide synthase

NOD	nucleotide-binding oligomerization domain-containing protein
NSM	neurosphere media
$p75^{NTR+}$	tumor necrosis factor receptor superfamily member 16
PGN	peptidoglycan
pH3	phospho-histone H3
RET	receptor tyrosine kinase
SEAP	secreted embryonic alkaline phosphatase
S.E.M.	standard error of the mean
SPF	specific pathogen free
TH	tyrosine hydroxylase
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor alpha
TPH2	tryptophan hydroxylase 2
TrkC	tropomyosin receptor kinase C
TUNEL	TdT-mediated dUTP nick end labeling

STATEMENT OF CONTRIBUTIONS

This project was initiated by Dr. Elyanne Ratcliffe. Funding for the experiments was provided by NSERC, which was also obtained by Dr. Elyanne Ratcliffe. Rajka Borojevic and Julia Bandura contributed to the establishment of the *in vitro* model. Dr. Varun Anipinidi assisted with the flow cytometry. Experimental advice was provided by Dr. Elyanne Ratcliffe, Dr. Laurie Doering, and Dr. Jane Foster, who were members of Jelena Popov's committee. Establishment of experimental protocols, isolation and culturing of cells, preparation of cells for flow cytometry, immunofluorescence, and quantification by immunofluorescence were performed by Jelena Popov. This thesis was written by Jelena Popov, with guidance from Dr. Elyanne Ratcliffe.

1. INTRODUCTION

1.1 Overview of the Gastrointestinal Tract

The gastrointestinal (GI) tract is responsible for movement and digestion of food, secretion of water and digestive enzymes, absorption of key nutrients, excretion of waste, and protection against pathogens and chemicals (Obata & Pachnis, 2016; Furness et al., 2013). These important physiological roles are mediated by the mucosal immune system, the enteric nervous system (ENS), and signaling hormones and peptides produced by enteroendocrine cells (Obata & Pachnis, 2016; Furness et al., 2013; Furness, 2008). Integrated communication between these different systems is essential for the maintenance of barrier function, selectivity of uptake of digested nutrients, and prevention of pathogenic invasion and inflammation (Furness et al., 2013; Foster et al., 2013). Defects in these interactions become evident in multiple gastrointestinal pathologies, including: inflammatory bowel disease (IBD), irritable bowel syndrome, Hirschsprung's disease, and intestinal pseudo-obstruction (Powell et al., 2017). While intestinal disorders have primarily been attributed to genetic defects, recent research has evoked a growing appreciation for the potential role of environmental factors on normal GI functioning and development.

1.2 Enteric Neural Crest Derived Cells and ENS Development

ENS development demands tight regulation of precursor cell differentiation, neurite growth, and establishment of neural networks. Colonization of the gut is mediated by two populations of enteric neural crest derived cells (ENCDCs) with unique patterns of

migration. The majority of ENS precursors originate from the vagal neural crest, invade the foregut, and migrate rostra-caudally and cluster at the cecum. Pre-ENCDCs of neural crest origin are guided by glial cell line-derived neurotrophic factor (GDNF) expression and give rise to most of the ENS (Young et al., 2014; Lake & Heuckeroth, 2013). In contrast, sacral ENCDCs have delayed entry into the gut, display caudo-rostral migration mediated largely by endothelin (ET3) expression, and innervate the distal portion of the hindgut (Miyahara et al., 2011; Lake & Heuckeroth, 2013). In mouse development, vagal ENCDCs first invade the foregut mesenchyme at E9.5 (corresponding to week 4 in human development). Linear migration is complete by E14.5 (week 7 in human development), and ENCDCs commence inward, radial migration and differentiation, giving rise to the myenteric plexus (Uesaka et al., 2016). It is around this time, at E15.5 (approximately week 8 in human development), that ENCDCs are most abundant (Binder et al., 2015).

Subsequent ENCDC invasion gives rise to the submucosal plexus and promotes extrinsic innervation (Uesaka et al., 2016). While myenteric ganglia reside throughout the entirety of the GI tract (from the esophagus to the rectum) and consist of longitudinal and circular muscle layers, the submucosal ganglia, in contrast, are restricted to the small and large bowel (Powell et al., 2017).

1.3 The Role of Growth Factors, Transcription Factors, and Chemoattractants in ENS Development

As ENCDCs migrate and innervate the gut rostro-caudally, those located at the proximal end of the gut begin to differentiate into neurons. This constitutes the act of gut

innervation, establishment of ganglia, and results in the emergence of the ENS. Continued proliferation toward the distal end of the gut is critical and must continually happen to overcome the differentiation occurring higher up the migrating crest (Burns et al., 2000). Therefore, the speed of proliferation in comparison to speed of differentiation is critical in ensuring proper innervation of the ENS. This prenatal establishment of the ENS involves a complex interplay between a variety of transcription factors, proteins, growth factors, and chemoattractants with variable locations and concentrations of expression in the developing gut. These have all been hypothesized to play a role in determining ENCDC fate (Harrison & Shepherd, 2013).

Among these many factors, bone morphogenetic factor (BMP) has been found to induce sympathetic neuron formation and noradrenergic differentiation (Morikawa et al., 2009). BMP exhibits a concentration gradient and it is hypothesized that this gradient might influence precursor differentiation based on exposure time and dose (Harrison & Shepherd, 2013). Wnt exhibits similar differences in concentration along the developing GI tract and has been shown to stimulate development of sensory neurons (Lee et al., 2004). One of the earliest transcription factors to be expressed in the developing ENS and a marker of ENCDC state is Sox10 (Elworthy et al., 2005; Young et al., 2002). Sox10 has been shown to play a critical role in influencing ENS development through: inducing receptor tyrosine kinase (RET) expression, inducing (endothelin receptor type B) EDNRB expression, inducing Phox2B expression, and through these interactions, ultimately maintaining the ENCDC progenitor state during neurodevelopment and even into adulthood (Lang & Epstein, 2003; Zhu et al., 2004; Elworthy et al., 2005; Harrison & Shepherd, 2013). Two

notable pathways for ENS innervation include the GDNF/RET/GFRa1 signaling pathway and the ET3/EDNRB signaling pathway.

The GNDF receptor complex is composed of receptor tyrosine kinase, RET, and ligand-binding co-receptor, GRFa1, both of which are expressed by ENCDCs and mediate neural guidance and maturation (Young et al., 2001). GDNF, a chemoattracted for vagal ENCDCs, is expressed by the gut mesenchyme and mediates rostro-caudal migration of enteric neural precursors throughout the gut (Young et al., 2001).

Aberrant signaling through mutant receptors or GDNF inactivation prevents proper innervation of the distal gut. This disruption of the GDNF signaling pathway typically manifests in Hirschsprung's disease, a congenital disease characterized by regions of colonic aganglionosis, mainly affecting the distal rectum. The affected region extends proximally, and the extent of disease involvement is dependent upon the location of disruption of the GDNF signaling pathway (O'Donnell & Puri, 2010). Hirschsprung's disease arises from the inability of migrating ENCDC populations to meet properly to form a cohesive neural network (O'Donnell & Puri, 2010).

GDNF concentration changes throughout embryonic development. Initially, GDNF concentration is highest in the foregut, and eventually increases toward the cecum as the wave of ENCDCs follows. ET3 serves as an important chemoattractant for ENCDCs past the cecum (Sasselli et al., 2012). Some research has assessed the role of ET3 in promoting ENCDC migration to the hindgut as it is specifically expressed within the cecum (Nagy & Goldstein, 2006). Support for this theory comes from *in vitro* experimental evidence by Nagy & Goldstein (2006) who showed that excess ET3 results in significantly increased

ENCDC proliferation and increased number of ganglionic cells. In contrast, inhibition of EDNRB results in severe hypoganglionosis (Nagy & Goldstein, 2006). Additional research in E12 Is/Is (ET3 deficient) mice provided with ET3 demonstrated ENCDC migration and colonization of the distal aganglionic gut (Wu et al., 1999). Further, *in vitro* inhibition of EDNRB receptor results in loss of "chain migration" of ENCDCs (Druckenbrod & Epstein, 2009). These findings stress the importance of input from the microenvironment in influencing ENS development.

1.4 Developmental Timeline of Enteric Neuron Maturation

The prenatal and perinatal periods of ENS development are characterized by rapid migration and proliferation of ENCDCs as well as terminal differentiation into the many neuronal subtypes that ultimately constitute a mature ENS.

Serotonergic, or 5-hydroxytryptamine (5-HT), neurons form a small subset of enteric neurons and are responsible for affecting small intestinal motility (Neal et al., 2009). They are among the first neural subtype to arise and coincide with the first wave of ENCDC invasion of the foregut at E9.5. The birth date of serotonergic neurons is typically complete by E15.5 (Li et al., 2011) (Figure 1A).

Nitrergic, or nitric oxide synthase (nNOS), neurons comprise a heterogenous mixture of approximately 90% circular muscle inhibitory neurons and 10% interneurons (Qu et al., 2008; Lake & Heuckeroth, 2013). This functional heterogeneity is reflected in the variable birthdating of nitrergic populations: while a subset of early born nitrergic neurons mirrors the appearance of serotonergic neurons with slow emergence beginning at

E11.5 and peaking at E15.5, a separate subset continues to develop into the postnatal period with cell cycle exit at P10.5 (Bergner et al., 2014) (Figure 1B).

In contrast, late born neurons such as dopaminergic and CGRP neurons exhibit delayed development and may continue differentiating for several weeks into the postnatal period. Dopaminergic neurons, for example, begin differentiating soon after serotonergic neurons arise in the perinatal period and exit the cell cycle at approximately P10.5 (Chalazonitis et al., 2008; Bergner et al., 2014) (Figure 1C). CGRP-expressing neurons also exhibit later birthdating, with contradictory evidence suggesting cell cycle emergence starting from E10.5 to a timepoint between E18.5 to P5.5 (Bergner et al., 2014; Pham et al., 1991). Interestingly, development and activity of both, dopaminergic and CGRP neurons, has been shown to be influenced by 5-HT release from serotonergic neurons. Dopaminergic neurons seem to play a role in mediating intestinal motility and preventing colonic hypermotility, although their exact role remains unclear (Li et al., 2006; Serio et al., 2011). Previous research has demonstrated that the presence of 5-HT influences the proportion of dopaminergic neurons in culture (Li et al., 2011). CGRP neurons play an important role in the peristaltic reflex by stimulating ascending contractions and descending relaxation (Grider et al., 1996). It has been demonstrated that 5-HT stimulates respective receptors on CGRP neurons, and administration of a CGRP antagonist inhibits this activity (Grider, 2003). This extended period for neurogenic potential is a unique characteristic of the ENS (Uesaka et al., 2015).

1.5 The Intestinal Microbiome

The gut lumen is home to an estimated 10^{14}-10^{15} bacteria, predominantly from the phyla Bacteroides and Firmicutes, and to a lesser extent Actinobacteria, Proteobacteria, and Verrucomicrobia (Donaldson et al., 2016). Recent research has suggested that the intestinal microbiome can be categorized into three distinct clusters or enterotypes that are defined by the dominance of one specific bacterial cluster in a host. The bacterial populations that define these three most common enterotypes has been controversial; some researchers have suggested clustering into Bacteroides, Prevotella, and Ruminococcus, while others suggest clustering into one Bacteroides and two Prevotella (Arumugam et al., 2011; Wu et al., 2011). While changes to relative proportions and species can be observed within hours of undertaking drastic dietary changes, enterotypes remain stable, suggesting enterotypes are less mutable to transient dietary and environment changes, and play a role in establishing long-term gut microbial composition (Wu et al., 2011). The existence of these robust enterotypes across individuals may further suggest that there may be a small number of host-microbial symbiotic states (Arumugam et al., 2011).

There is a distinct species-density and overall bacterial concentration gradient throughout the gastrointestinal tract, with increasing bacterial load from the small bowel towards the colon and rectum. These differences coincide with changes in luminal pH, oxygen concentrations, and presence or absence of antimicrobial peptides (such as bile acids). These factors are recognized to be conducive to specific bacterial species' survival (Donaldson et al., 2016).

Until recently, it has been believed that the prenatal environment is sterile (Jimenez et al., 2008). While parturition and infancy constitute the first largest colonization of gut bacteria, recent evidence suggests that exposure to even simple flora in the perinatal period can affect early life ENS structure and function (Collins et al., 2014). The gut microbiota continues to mature after this initial colonization, and these changes in gut microbiota throughout childhood are characterized by a reduction in aerobic and facultative anaerobic bacteria and an increase in anaerobic bacteria (Borre et al., 2014). These gradual changes in bacterial composition continue into adolescence, along with diversification of the microbiota to resemble an adult microbiome. During these developmental milestones, changes in gut microbiota mirror changes immune system function, brain development, and ENS development (Jasarevic et al., 2016).

1.5.1 Intestinal Microbiota and the Immune System

Specific bacterial components such as lipopolysaccharide (LPS), flagellin, and peptidoglycan (PGN) derivatives, muramyl dipeptide (MDP) and gamma-D-Glu-mDAP (iE-DAP), have been shown or have been postulated to influence the developmental potential of enteric neurons, activate immune cells, and promote the onset of acute and chronic inflammatory conditions. The proposed pathophysiology involves enteroendocrine cell recognition of luminal LPS and flagellin, followed by enteroendocrine cell release of chemokines, C-X-C motif chemokine ligand 1/3 (CXCL1/3) and C-C motif chemokine ligand 20 (CCL20), that attract immune cells from the lamina propria (Selleri et al., 2008). These immune cells are then activated by interleukin 32 (NK4/IL-32) binding to

nucleotide-binding oligomerization domain-containing protein 2 (NOD2). Ultimately, this results in activation of the kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory pathway, and increased production of tumor necrosis factor alpha (TNF-α) and inflammation (Selleri et al., 2008). This is supported by findings that NK4/IL-32 are increased in chronic inflammatory conditions such as Crohn's disease and psoriasis, with expression levels correlating with severity of disease (Dinarello & Kim, 2006; Shioya et al., 2007).

1.5.2 Intestinal Microbiota and Behavior

The adult microbiome can also be susceptible to changes and these changes may be reflected in behavior. Bercik and colleagues (2011) found that administration of oral antimicrobials transiently influenced changes in the intestinal microbiota of specific pathogen free (SPF) mice 8-10 weeks of age, which were normalized two weeks after antimicrobial exposure (Bercik et al., 2011). This was mirrored by equally transient changes in hippocampal brain-derived neurotrophic factor (BDNF) expression and exploratory behavior. The same experimental procedure was not able to replicate these findings in germ free (GF) mice. Further, gavage feeding adult GF NIH Swiss mice with cecal microbiota from timid SPF BALB/c mice resulted in substantially decreased exploratory behavior compared to colonization with cecal contents from the more exploratory SPF NIH Swiss mice. Similarly, gavage feeding of adult GF BALB/c mice with cecal SPF microbiota from exploratory NIH Swiss mice resulted in substantially increased exploratory behavior compared to colonization with SPF microbiota from the

more timid BALB/c mice (Bercik et al., 2011). Taken together, these findings demonstrated that the interaction between intestinal microbiota and the immune system, brain development, and behavior is plastic. Furthermore, communication between the gut and brain is bi-directional. Microbial-derived neuromodulatory and structural metabolites influence brain activity through pathways involving the vagus nerve and intestinal epithelial-expressed immune receptors. The brain also exerts a *top-down* influence on the gut microbiome, shaping the bacterial community structure via hormonal control through the hypothalamic-pituitary axis (HPA) and systemic inflammatory responses (Bauer et al., 2016).

1.5.3 Intestinal Microbiota and the ENS

The ENS serves as a key relay point for the gut-brain axis, mediating interactions between nutrients and microbes of the external environment within the gut lumen, and immune cells and cellular contents of the internal environment (Obata & Pachnis, 2016; Lake & Heuckeroth, 2013). The development of the ENS begins during the prenatal period, and maturation is thought to be affected by the microbiome throughout the postnatal period. During this developmental period, the ENS continues to interact with, and be shaped by, the internal microenvironment and the external environment (Powell et al., 2017). Further, the perinatal period experiences the greatest diversification of the intestinal microbiome (Borre et al., 2014). From the early postnatal period to adolescence, these systems continue to mature and are influenced by internal and environmental factors.

The microbiome may play a role in ENS structure and function as early on in life as postnatal day 3 (Collins et al., 2014). In the early postnatal period, NIH Swiss GF mice exhibit significantly decreased nerve fiber densities with irregular spacing between nerve fibers compared to SPF and altered Schaedler flora (ASF) mice in the jejunum and ileum. Furthermore, GF mice exhibit significantly decreased myenteric neurons per ganglia and increased nitrergic neurons per ganglia. These structural aberrancies are mirrored by functional changes in small intestinal motility, including decreases in frequency and amplitude of circular muscle contractions which were not affected by voltage-gated sodium channel blockage with lidocaine, in contrast to SPF mice and ASF mice (Collins et al., 2014). These findings demonstrated that even the simple intestinal flora present in ASF mice may be sufficient in mediating the proper development of the ENS.

1.5.4 Role of Lipopolysaccharide in the Gastrointestinal Tract

Traditionally, it was believed that the ENS and microbiome interact indirectly through immune mediators; however, increasing evidence suggests that myenteric neurons may express TLR4. Furthermore, myenteric neurons and ENCDCs may interact directly with and be responsive to LPS with concentration-dependent increases in the NF-κB inflammatory pathway, although the fate of such interactions remains contradictory (Anitha et al., 2012; Arciszewski et al., 2008).

LPS is a gram-negative endotoxin that signals via transmembrane protein TLR4 and activates inflammatory cytokines via the NF-κB pathway. Anitha and colleagues (2012) studied the role of TLR4 signaling in enteric neuronal survival by using *TLR4-/-*, $Tlr4^{Lps\text{-}d}$,

and *Myd88-/-* mice (Anitha et al., 2012). *Tlr4*$^{Lps\text{-}d}$ mice exhibited a significant decrease in weight compared to wildtype mice. *Tlr4*$^{Lps\text{-}d}$ mice also exhibited decreased intestinal transit and colonic motility, demonstrated by relative distribution of fluorescein isothiocyanate (FITC) fluorescence and bead expulsion time, respectively. Their findings also suggested that LPS promotes ENCDC survival, maturation, and gut motility through TLR4/MyD88. Furthermore, they demonstrated dose-dependent activation of NF-κB in ENCDC cultures (Anitha et al., 2012), although the exact mechanisms involved remain unclear.

In contrast to the findings by Anitha and colleagues (2012), Arciszewski and colleagues (2008) demonstrated increased neurodegeneration in the presence of high concentrations of LPS in adult rat myenteric neuron cell cultures (Arciszewski et al., 2008). This was postulated to be due to LPS-induced neuronal hyperexcitation that leads to excitotoxicity through excitatory transmitters such as glutamate (Arciszewski et al., 2008). Interestingly, this neuronal cell loss has been replicated in experimentally induced colitis, suggesting that IBD may be mediated by LPS-induced neurodegeneration and reduction in neuronal plasticity (Arciszewski et al., 2008). Furthermore, insults to the gut that compromise the intestinal epithelial barrier may result in ENS interactions with the bacterial products, followed by disruptions in the structure and function of the ENS.

The discrepancy in the results between these two studies may be explained by the differences in LPS structure isolated from different bacterial species which determines their immunogenicity (Steimle et al., 2016). While LPS has been identified as one of the most potent immune stimulators, its immunostimulatory abilities vary depending on the number of lipid A acyl chains, as well as the number of phosphate groups. In general, the strength

of the LPS agonist increases with larger lipid A acyl chains (with hexa-acyl being more potent than penta-acyl) and increased phosphate groups (two being stronger than one, or none) (Steimle et al., 2016).

Alternatively, these differences may also be due to the maturation states of the neurons. Anitha and colleagues (2012) studied LPS effects on ENCDC cultures, while Arciszewski and colleagues (2008) analyzed cultures comprised of adult myenteric neuron. There is evidence to suggest that apoptosis is not a characteristic of ENCDCs but does occur in mature myenteric neurons (Gianino et al., 2003; Kulkarni et al., 2017).

While the effects of LPS on enteric neurons remain controversial, previous research has demonstrated substantial TLR4 expression in glia and neurons of the myenteric and submucosal plexuses throughout the intestine, with highest localization in the distal large bowel in adult mice (8-32 weeks of age) (Barajon et al., 2009). Given that enteric neurons extend their projections to the intestinal epithelial layer, it is reasonable to hypothesize that they interact with TLR4 ligands such as LPS within the gut lumen, and in this manner, mediate tolerance to commensal microbiota (McVey Neufeld et al., 2013). TLR4 expression by myenteric neurons has not yet been explored in prenatal or early postnatal mice, nor has it been quantified.

1.5.5 Role of Flagellin in the Gastrointestinal Tract

Flagellin is a structural component of the flagella filaments, allowing certain bacteria motility. It is highly conserved across Gram-negative and Gram-positive bacteria. Flagellin is recognized by the TLR5 receptor and activates the NF-κB pathway through

proinflammatory cytokines such as TNF- α (Lopez-Yglesias et al., 2014; Gewirtz et al., 2001). Flagellin has specifically been associated with the production of chemokines and antimicrobial compounds in neutrophils, monocytes, and epithelial cells (Shibata et al., 2012; Aziz et al., 2013). Flagellin has also been shown to induce dendritic cell maturation and promote dendritic cell migration into the lumen for phagocytosis by opening epithelial tight junctions (Shibata et al., 2012; Shi et al., 2017; Rescigno et al., 2001).

1.5.6 Role of Muramyl Dipeptide and iE-DAP in the Gastrointestinal Tract

Muramyl dipeptide is a bacterial wall and peptidoglycan component of both, Gram-negative and Gram-positive bacteria, recognized by NOD2. It induces IL-1α and IL-6 cytokine production via nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) and IL-32 in colonic epithelial tissue, ultimately resulting in NF-κB activation and inflammation (Netea et al., 2005; Inohara et al., 2003). Mutations in NOD2 and inability to recognize MDP have been shown to play a role in intestinal inflammation and susceptibility to Crohn's disease (Saem et al., 2013). iE-DAP is also a dipeptide component of peptidoglycan, and the minimal motif recognized by NOD1. NOD1 is an intracellular sensor, expressed in several tissue types. MDP and iE-DAP are both components of peptidoglycan. Together, they result in oligomerization of receptors NOD1 and NOD2, and activation of the NF-κB pathway (Strober et al., 2006).

While communication between the microbiome and host nervous system is essential for proper development and function of the ENS, this interaction is complex and not fully understood. The influences of LPS, flagellin, MDP, and iE-DAP on ENCDC fate and ENS

development remain unclear. Furthermore, it is unknown whether these bacterial products exert their effects on enteric neurons directly, or indirectly via the epithelium, immune cells, or other intermediates.

1.6 Clinical Relevance

Previous research has analyzed the interactions between intestinal microbiota and the immune system, neurodevelopment, and behavior. Maternal exposure to antibiotics during pregnancy has been associated with increased intestinal permeability and altered immune responses in the offspring (Fak et al., 2008; Lamouse-Smith et al., 2011). Furthermore, infants exposed to intrapartum antibiotics display changes in their stool microbiota prolife compared to controls (Azad et al., 2013; Schrag et al., 2000). These findings suggest that not only does microbial colonization occur in the prenatal period, but the fetal microbiome may also be susceptible to antibiotic exposure which may alter proper development of the GI tract. This is particularly relevant as it has been reported that up to 30% of North American infants are exposed to intrapartum antibiotics as a results of maternal group B streptococcus status (Spaetgens et al., 2002; Verani et al., 2010). While it has been recognized that genetic factors play a role in ENS development, evidence suggests that the intestinal microbiome may also play an important role.

Based on these findings, it is now important to understand how perturbations in early life microbial colonization could affect the ENS development.

1.7 Thesis Hypothesis and Objectives

Hypothesis

The aim of this project was to construct an *in vitro* model of ENCDCs through immunoselection using ENCDC marker tumor necrosis factor receptor superfamily member 16 ($p75^{NTR+}$). The $p75^{NTR+}$ receptor contains an extracellular domain and is a marker of neural progenitors (Binder et al., 2015). Once established, the *in vitro* ENCDCs would serve as a platform for assessing whether key bacterial products of the intestinal microbiome have direct effects on ENS development. This would be assessed by determining baseline culture characteristics, and comparing to changes in proliferation, apoptosis, and differentiation post incubation with the bacterial products. It is hypothesized that bacterial products influence the developmental programming of enteric neurons.

Objectives

The overarching theme of this project was to investigate the effects of intestinal microbiota on influencing ENS development. This project addressed the following specific aims:

Specific Aim 1: To establish an *in vitro* model of ENCDCs and to characterize the chemical coding of cultured ENCDCs.

Specific Aim 2: To determine whether bacterial products have an effect on cultured ENCDC proliferation, differentiation, and apoptosis.

2. MATERIALS & METHODS

2.1 Animals

Timed pregnant SPF mice on a C57Bl/6NTac background (inbred strain) were ordered from Taconic Biosciences, Inc. (Rensselaer, NY, USA). SPF mice were maintained on ventilated racks in ultraclean units in the McMaster Central Animal Facility. The day of plug detection was considered E1. Timed pregnant dams (n = 6) were sacrificed at E15.5 under sterile conditions. Mice were anaesthetized using isoflurane and sacrificed by cervical dislocation. Treatment of animals and all experiments were conducted in accordance with a project approved Animal Utilization Protocol and the McMaster Animal Research Ethics Board.

2.2 ENCDC Isolation

Timed pregnant SPF dams were sacrificed at E15.5, and fetuses (n = 48) were extracted under sterile conditions in a biological safety cabinet. Entire fetal guts were isolated, and digested with collagenase (1 mg/ml, Sigma C0130) and manual trituration using glass pipettes. Cells were passed through a cell strainer (Invitrogen 352340) to ensure single cell preparation. Single cells were incubated with: primary antibody rabbit anti-mouse $p75^{NTR+}$ (1:50, Alomone ANT-007), and secondary antibody anti-rabbit IgG microbeads (150 µl per 10^7 cells; MACS Miltenyi Biotec 130-048-602). Cells were passed through a magnetic cell separation column (MACS Miltenyi Biotec 130-042-202). Negative cells were discarded. Positive cells were plated on 2% fibronectin (Sigma F1141) coated plates at a density of approximately 275,000 cells/ml in 6 well plates. Cells were

incubated at 37°C, 5% CO_2. Neurosphere media (NSM+) supplemented with N2, B27, epidermal growth factor (EGF; recombinant, mouse), basic fibroblast growth factor (bFGF; human), heparin, and antibiotics streptomycin/penicillin (Table 1). The growth factors and nutrients used in this project are common components of neuron media. NSM+ media was supplemented with 10% horse serum for the first 24 hours after dissection to facilitate adherence. After 24 hours, the media was changed to standard NSM+ media with nutrients and growth factors only. Old NSM+ media was replaced with fresh media every 3-4 days. Adherent cells were dissociated using Accutase (Invitrogen 1110501) approximately every 14 days and reseeded at the same initial density to form a new subculture. Cell proliferation and viability were assessed by counting using a hemocytometer (Sigma Z359629), trypan blue dye (Invitrogen 15250-061), and an Olympus CX331 binocular microscope (Olympus Corporation, Tokyo, Japan) at each passage.

2.3 Flow Cytometry

Cultured cells were processed for flow cytometry at the third and fourth subcultures to determine purity of target $p75^{NTR+}$ cells. We were unable to perform such experiments prior to the third subculture as sufficient cells were not present. Allowing several passages before assessing the proportion of $p75^{NTR+}$-expressing cells concurrently enabled testing of our culture conditions for the ability to maintain ENCDC in an undifferentiated state. Cultured cells were stained with $p75^{NTR+}$-FITC (Alomone Labs ANT-007F) for detection of ENCDCs, and viability dye 7-aminoactinomycin D (7-AAD; Invitrogen 00-6993-50) for assessment of live cells. All samples were run using the BD FACSCanto flow cytometer

(San Jose, CA, USA) connected to a Hewlett-Packard computer (Hewlett-Packard Inc., Palo Alto, CA, USA). Data acquisition and analysis was performed using FACSDiva (BDFACSDiva, Aukland, New Zealand) and FlowJo softwares (FlowJo LLC, Ashland, OR, USA).

After dissociation with Accutase, cells were washed in 1 ml of flow cytometry buffer in polystyrene tubes, composed of 0.2% bovine serum albumin (BSA) dissolved in 1% phosphate-buffered saline (PBS; pH 7.4). Fc block (BDBiosciences 553141) was added to prevent nonspecific antibody binding to the Fc receptor of any potential leukocytes (Andersen et al., 2016). Cells were stained with 100 µl of antibody solution for ENCDC marker, $p75^{NTR+}$-FITC (1:10, Alomone Labs ANT-007F), and were incubated on ice, in the dark, for 30 minutes. Samples were subsequently labeled with 7-AAD to assess cell viability. One tube was prepared in flow cytometry buffer alone as the unstained control. An additional tube was prepared using the antibody and OneComp eBeads™ Compensation Beads (Invitrogen 01-1111-41). Samples were washed and resuspended in 400 µl of flow cytometry buffer and passed through polystyrene filter top tubes to remove cell aggregates. Samples were immediately run on the cytometer without fixing. All sample tubes were prepared containing 10^6 cells.

2.4 Cryopreservation of Cultures

Cultured cells were cryopreserved after the fourth subculture in NSM+, 10% dimethyl sulfoxide (DMSO; Sigma D2438). Cells were frozen at a density of $1x10^7$ at approximately -1°C/minute (Mr. Frosty™, Invitrogen 5100-0001) in a -80°C freezer. After

24 hours of cooling, cells were transferred to the vapor phase of liquid nitrogen for long-term storage.

2.5 Characterization of Cultures through Immunofluorescence

2.5.1 Preparation of Cultured Cells for Antibody Staining

Cultured cells were grown in chamber slides (Invitrogen 154526), at a density of approximately 275,000 cells/ml of NSM+ media, with or without varying concentrations of bacterial products. On the day of preparation for immunofluorescence, 0.5 ml of NSM+ was discarded, and 0.5 ml of freshly prepared 4% paraformaldehyde was added. After 2 minutes of fixing, the paraformaldehyde was discarded and replaced by 1 ml of freshly prepared 4% paraformaldehyde for an additional 5 minutes. Cells were washed in 1% PBS, pH 7.4, three times (5 minutes each wash) to remove residual paraformaldehyde. Cells were permeabilized by incubating in blocking buffer (1% PBS, 4% normal horse serum (Invitrogen 26050070), 0.5% Triton^{TM} X-100 (Sigma T8532)) for 1 hour. Subsequent incubations with primary and secondary antibodies allowed for visualization of target cells. All slides were sealed with Vectashield (Vector Laboratories VECTH1000) and coverslipped to minimize photobleaching. Antibodies against HuC/D were to confirm neuronal phenotype. Bisbenzimide was used as a counterstain to stain all nuclei present.

2.5.2 Antibody Staining for Quantification of ENCDCs

To assess culture purity of target ENCDCs, cells were stained with primary antibodies against p75^{NTR+} (1:50, Alomone Labs ANT-007), secondary donkey anti-rabbit

Alexa Fluor 594 (1:200, Molecular Probes A21207); primary HuC/D biotin (1:50, Invitrogen A21272), secondary streptavidin 488 (1:200, Molecular Probes S11223); and bisbenzimide (1 µg/ml, Sigma H33258). Staining with secondary antibody alone was done as a control.

2.5.3 Antibody Staining for Quantification of Serotonergic, Nitrergic, Dopaminergic, and Proliferating Neurons

Cells were assessed for differentiation into serotonergic, nitrergic, and dopaminergic neurons by staining for antibodies against 5-HT, nNOS, and tyrosine hydroxylase (TH), respectively. TH is the rate-limiting enzyme of catecholamine biosynthesis, responsible for converting tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). Subsequent reactions convert L-DOPA to dopamine, norepinephrine, and epinephrine (Colette Daubner et al., 2012). While noradrenergic and adrenergic neurons extend projections that innervate the gut, the cell bodies of these neurons are extrinsic and located outside of the ENS (Li et al., 2010). Norepinephrine is produced by dopamine β-hydroxylase within vesicles. Dopaminergic enteric neurons are unique in that they do not express the dopamine β-hydroxylase enzyme and are therefore unable to produce norepinephrine or epinephrine (Serio et al., 2011). Instead, cell bodies supplying norepinephrine arise from the prevertebral ganglia and innervate the myenteric plexus via mesenteric nerves (Cooke, 1986). Further processing of norepinephrine by phenylethanolamine N-methyltransferase produces epinephrine within the adrenal medulla (Mittal et al., 2017; de Jonge, 2013). Therefore, immunolabeling of enteric neurons using

TH antibodies would be indicative of only intrinsic dopaminergic neurons (Nezami & Srinivasan, 2010; Li et al., 2010; Li et al., 2004).

Differentiation of cultured ENCDCs was assessed by staining cells with: primary antibodies to 5-HT (1:200, Immunostar 20080), secondary donkey anti-rabbit Alexa Fluor 594 (1:200, Molecular Probes A21207); primary rabbit anti-neuronal nNOS (1:500, Cedarlane), secondary donkey anti-rabbit Alexa Fluor 594 (1:200, Molecular Probes A21207); and primary TH (1:500, Millipore Sigma AB1542), secondary donkey anti-sheep Alexa Fluor 488 (1:200, Invitrogen A11015) or secondary donkey anti-sheep Alexa Fluor 594 (1:200, Invitrogen A11016). Total positive cells were manually counted and compared to vehicle control. Staining with secondary antibody alone was done as a control.

Proliferation of cultured ENCDCs was assessed by labelling with primary antibodies to phospho-histone H3 (pH3; 1:500, BioLabs 9705S), secondary donkey anti-goat Alexa Fluor 594 (1:200, Invitrogen A11058). Histones are heavily phosphorylated at serine 10 on metaphase chromosomes during mitosis. This phosphorylated state persists until exist from mitosis, enabling reliable measurement of proliferative cells (Hans & Dimitrov, 2001). Cells were manually counted and compared to vehicle control. Staining with secondary antibody alone was done as a control.

2.5.4 Detection and Quantification of Apoptotic Cells

Apoptosis of cultured ENCDCs was detected using TdT-mediated dUTP nick end labeling kit (Promega DeadEnd^{TM} Fluorometric TUNEL System G3250). This assay involves TdT binding to blunt ends of DNA fragments and catalyzing incorporation of

fluorescently labelled nucleotide, fluorescein-12-dUTP (Kyrylkova et al., 2012). Total apoptotic cells were manually counted and compared to vehicle control. Treatment with DNase (Promega M6101) was performed for the positive control, and omission of the terminal deoxynucleotidyl transferase-recombinant (rTdT) enzyme was done for the negative control.

2.5.5 Image Analysis

All images were coded to ensure investigator blinding of experimental conditions during image analysis. All data were analyzed using fluorescent microscope Leica DMRXA2 (Leica Microsystems Inc, Concord, ON, Canada) and Volocity software (Improvision Inc., Montreal, QC, Canada) on a Macintosh computer (Apple Computers, Markham, ON, Canada).

Quantification of ENCDC cultures was performed at 20x magnification using 5 standard fields. Quantification of ENCDCs treated with bacterial products and vehicle control was performed at 20x magnification using 5 standard fields.

2.6 Exposure to Bacterial Products

Cells were seeded on fibronectin-coated plastic chamber slides (Invitrogen 154526) at a density of approximately 270,000 cells/ml for establishment of the fifth subculture. Cells were incubated with NSM+ media for 24 hours to facilitate adherence prior to treatment with bacterial products.

ENCDC cultures were incubated with LPS (0.001, 0.01, 0.1, 1 µg/ml, Sigma L2630), flagellin (0.001, 0.01, 0.1, 1 µg/ml, Enzo Life Sciences ALX-522-058-C010), MDP (0.01, 0.1, 1, 10 µg/ml, Invivogen tlrl-mdp), iE-DAP (0.1, 1, 10, 100 µg/ml Invivogen tlrl-dap), and NSM+ vehicle control. Varying concentrations of bacterial products were used to assess concentration dependent effects. Bacterial product solutions were prepared in fresh NSM+ media. Cells were incubated for 24 hours prior to fixing in 4% paraformaldehyde and processing for proliferation and chemical coding by immunofluorescence, and apoptosis by the TUNEL assay.

2.7 Statistical Analyses

Statistical analyses were done using a Mann-Whitney U with comparisons of culture characteristics between subculture 4 and subculture 5. Data analyses of effects of bacterial products on cultured ENCDCs were performed using Kruskal Wallis with Dunn's post hoc test to determine significance between conditions and dose-dependent responses. Significant outliers were removed. The level of statistical significance was set at $p < 0.05$. Data are presented as mean ± S.E.M. All analyses were performed using GraphPad Prism Version 4 for Mac OS X (GraphPad Software Inc., La Jolla California, USA).

3. RESULTS

3.1 Characterization of ENCDCs in Culture

3.1.1 Expansion of ENCDCs

Cultures were assessed for proliferation and viability at each passage for five subcultures to determine whether NSM+ culture conditions were suitable for ENCDC growth (Table 2). A single subculture was grown for approximately two weeks after seeding. Old NSM+ media was replaced with fresh NSM+ every 3-4 days.

Cell proliferation was assessed at each passage immediately after dissociation using a hemocytometer and microscope. Cultures maintained high proliferative potential across all subcultures. Viability was assessed using trypan blue dye, and results were comparable to flow cytometry and demonstrated approximately 100% viability at each passage.

3.1.2 Assessment of ENCDC Culture Purity and Viability using Flow Cytometry

Flow cytometry was used to quantify the proportion of ENCDCs in the cultures by staining with antibodies against $p75^{NTR+}$ and 7-AAD viability dye. Analysis of cells from the third subculture revealed a 95.1% $p75^{NTR+}$-positive population with a 97.3% viability. Similar findings were observed on a repeat run of cells from the fourth subculture, demonstrating 96.1% $p75^{NTR+}$-positive cells and a 98.2% viability (Figure 2).

3.1.3 Characterization of ENCDC Cultures Using Immunofluorescence

Cultures were processed for immunofluorescence at the fourth subculture to further validate target cell purity and determine baseline culture characteristics. This was assessed

by manual counting of proportion of HuC/D expressing neurons coexpressing $p75^{NTR+}$ as a marker of immature neuronal state. These results were compared to total cells present in culture by staining with nuclear dye, bisbenzimide (Figure 3). This method was not appropriate for assessing cell viability; however, viability was assessed at every passage (every 2 weeks) using trypan blue dye.

Cells co-expressing $p75^{NTR+}$ and HuC/D composed the largest population (99.3% ± 1.18%). These baseline culture conditions also showed the presence of nitrergic neurons (5.83% ± 1.77%), and a smaller proportion of serotonergic neurons (1.60% ± 0.850%). No dopaminergic neurons were detected. Proliferating cells were also present (4.46% ± 1.56%).

3.2 Differentiation of ENCDCs into Early Born Neurons in Culture

Comparison of culture characteristics between subculture 4 and subculture 5 demonstrated a significant increase in proportion of serotonergic neurons (subculture 4, 2.00% ± 0.780% vs subculture 5, 8.76% ± 1.86%; $p = 0.0079$) and nitrergic neurons (subculture 4, 2.49% ± 0.890% vs subculture 5, 10.0% ± 2.96%; $p = 0.0079$) (Figure 4). No significant differences were found in the proportion of dopaminergic neurons (subculture 4, 0.00% ± 0.00% vs subculture 5, 0.180% ± 0.410%; $p > 0.05$) or proliferation (subculture 4, 2.53% ± 0.360% vs subculture 5, 3.52% ± 1.29%; $p > 0.05$).

3.3 ENCDC Cultures Treated with Bacterial Products

Cultured ENCDCs were incubated with varying concentrations of LPS, flagellin, MDP, iE-DAP, and vehicle control for 24 hours. All conditions were done in duplicates with the exception of TUNEL flagellin data and vehicle control.

3.3.1 LPS Effects on ENCDC Programming

LPS treatment demonstrated an increase in proliferation of ENCDCs at 0.1 µg/ml, compared to vehicle control ($p = 0.0405$) and 0.001 µg/ml ($p = 0.0242$) (Figure 5).

A dose-dependent increase in differentiation into serotonergic neurons (0.01 µg/ml, $p = 0.0024$; 0.1 µg/ml, $p < 0.0001$; 1 µg/ml, $p = 0.0085$), compared to the lowest does of LPS (0.001 µg/ml) was observed; however, there was no significant difference compared to vehicle control ($p > 0.05$).

There was a dose-dependent increase in differentiation into nitrergic neurons (0.01 µg/ml, $p = 0.0035$; 0.1 µg/ml, $p < 0.0001$; 1 µg/ml, $p = 0.0009$) compared to the lowest concentration of LPS (0.001 µg/ml), but not vehicle control ($p > 0.05$). There was a significant increase in nitrergic neurons between 0.1 µg/ml LPS compared to vehicle control ($p = 0.0198$).

There was no significant difference in apoptosis or proportion of dopaminergic neurons at any concentration of LPS tested.

3.3.2 Flagellin Effects on ENCDC Programming

Flagellin demonstrated a dose-dependent increase in proliferation compared to vehicle control at 0.1 µg/ml ($p = 0.0189$) and 1 µg/ml ($p = 0.0003$) (Figure 6). Dose-dependent increases in proliferation were also observed between 0.001 µg/ml and 0.1 µg/ml ($p = 0.0497$), 0.001 µg/ml and 1 µg/ml ($p = 0.0003$), and 0.01 µg/ml and 1 µg/ml ($p = 0.0173$).

An increase in proportion of serotonergic neurons was observed at 0.1 µg/ml ($p = 0.0074$) and 1 µg/ml ($p = 0.0055$) compared to vehicle control.

There was a significant increase in proportion of nitrergic neurons at 1 µg/ml of flagellin compared to vehicle control ($p = 0.0132$).

There was no significant difference in the proportion of apoptotic cells or dopaminergic cells at any concentration of flagellin tested.

3.3.3 MDP Effects on ENCDC Programming

There was a significant increase in proportion of serotonergic neurons between 0.01 µg/ml and 1 µg/ml MDP ($p = 0.0178$) (Figure 7). There was no significance compared to vehicle control.

A significant increase in nitrergic neurons was found between vehicle control and 0.1 µg/ml MDP ($p = 0.0038$), and vehicle control and 1 µg/ml MDP ($p = 0.0401$).

There was no significant difference in proportion of proliferating, apoptotic, or dopaminergic cells at any concentration of MDP tested.

3.3.4 iE-DAP Effects on ENCDC Programming

There was a significant increase in proportion of serotonergic neurons between vehicle control and 10 µg/ml iE-DAP ($p = 0.0233$) (Figure 8). This increase was also found between 1 µg/ml iE-DAP and 10 µg/ml iE-DAP ($p = 0.0341$).

There was a significant increase in proportion of nitrergic neurons between vehicle control and 0.1 µg/ml iE-DAP ($p = 0.0151$), and vehicle control and 1 µg/ml iE-DAP ($p = 0.0153$).

There was no significant difference in proportion of proliferating, apoptotic, or dopaminergic cells at any concentration of iE-DAP tested.

4 DISCUSSION

Increasing research is demonstrating the important role of the intestinal microbiome on human health, and specifically, on the development of the ENS. This project aimed to establish an *in vitro* model of ENCDCs to assess the direct role of bacterial products on ENS development. Subsequent experiments revealed that direct interactions with key bacterial products may influence proliferation and chemical coding of ENCDCs, but not apoptosis.

4.1 Establishment of the *in vitro* ENCDC model

Establishment of an ENCDC cell line was complicated by the limited number of antibodies available for ENCDCs, as well as the limited area of research in ENS neurodevelopment. Few resources existed on how to construct such a model.

In this project, isolation of ENCDCs at E15.5 using the $p75^{NTR+}$ marker yielded highly pure cultures. Incubation with NSM+ media demonstrated consistently high proliferative potential across several subcultures, as well as the maintenance of an undifferentiated state for four subcultures.

4.1.1 Characterization of ENCDC Cultures

Characterization of ENCDC cultured was primarily performed through flow cytometry on two separate occasions using the third and fourth subcultures. On both occasions, a substantial proportion of the cell population expressed $p75^{NTR+}$, indicative of nearly pure cultures (95.6% ± 0.710%). Both subcultures also demonstrated a high cell viability (97.8% ± 0.640%), indicating that the NSM+ media was suitable for ENCDC survival and growth.

We identified a total of 4.49% ± 1.67% differentiated cells in the fourth subculture, comprising entirely early born serotonergic and nitrergic neurons, the largest proportion of which were nitrergic neurons. This is in line with the notion that serotonergic neurons and a subset of nitrergic neurons constitute early born neurons, while dopaminergic neurons exhibit later birthdates (Pham et al, 1991).

The fifth subculture had an increase in differentiation, exhibiting a total of 18.9% ± 5.23% mature neurons. Once more, nitrergic neurons comprised the most abundant subpopulation (10.00% ± 2.96%), followed by serotonergic (8.76% ± 1.86%), and lastly dopaminergic neurons (0.180% ± 0.410%). Both, nitrergic and dopaminergic populations in subculture 5 are consistent with previously published reports on the proportion of these

neurons at P0 in C57Bl/6 mouse wholemounts. Bergner and colleagues (2014) demonstrated approximately 13.0% nitrergic neurons and approximately 0.880% dopaminergic neurons at P0 (Bergner et al., 2014). While Bergner and colleagues (2014) were unable to show the proportion of serotonergic neurons at P0, they did demonstrate an expansion from E15.5 (0.940%) to E18.5 (1.51%). These values were similar to our observations of the fourth subculture characteristics for serotonergic neuron proportions (2.00%); however, they were drastically lower than our observations in our fifth subculture (8.76%). Two possible explanations for this discrepancy exist.

Since Bergner and colleagues (2014) quantified neurons in freshly prepared wholemounts, while we performed analyses in an *in vitro* model, the first explanation might be that isolation of ENCDCs from their native microenvironment and deprivation of input from other systems may have impacted the terminal differentiation of these neurons (Ghallab, 2013).

The second explanation may involve the use of 5-ethynyl-2´-deoxyuridine (EdU) in quantifying neuron subtypes in the Bergner and colleagues (2014) study. EdU is a thymidine analogue that incorporates into DNA during S phase of the cell cycle, thus serving as a marker of proliferating cells (Bergner et al., 2014). As a result, EdU labelling would be strongest after the first cell cycle and would decrease with repeated proliferation. It is possible that the reason Bergner and colleagues (2014) reported such low levels of serotonergic neurons was due to these neurons either undergoing rapid proliferation and thus losing the marker quickly, or serotonergic neurons already having completed terminal

differentiation by the time they were stained, and therefore, minimal EdU was incorporated into the cells (Bergner et al., 2014).

While it is challenging to pinpoint the exact developmental timeline of our ENCDC cultures at the fifth subculture and just prior to treatment with bacterial products, based on the slow emergence of dopaminergic neurons, it is reasonable to assume that the fifth subculture was most representative of a perinatal timepoint. This differentiation into dopaminergic neurons likely commenced in the fifth subculture as the proportion (0.180%) was considerably less than the expected approximate proportion in the mature ENS (0.500%) or P0 ENS (0.880%) (Qu et al., 2008; Li et al., 2011; Bergner et al., 2014). These results may be suggestive of a heterogenous mixture of neuron subtypes between E15.5 to P0. Despite this, the relevance of the findings presented here should not be discredited based on the supposed heterogeneity as these populations still constituted a relatively immature ENS state. In addition, the consistencies between the relative proportions of differentiated neurons in this project and previously published reports further validates the ability to isolate highly pure populations of ENCDCs using immunoselection. Furthermore, the results from the fourth subculture which demonstrated a largely immature population of enteric precursors supports the ability to construct and maintain an undifferentiated ENCDC population in culture for future work.

4.1.2 Effects of Bacterial Products on the Chemical Coding of Cultured ENCDCs

Our results are suggestive of direct ENCDC interactions with LPS and flagellin in stimulating ENCDC proliferation and differentiation into early born neurons of

serotonergic and nitrergic subtypes. Peptidoglycan derivatives, MDP and iE-DAP, appear to mainly stimulate differentiation into nitrergic neurons, and in the case of iE-DAP also serotonergic neurons. MDP does not appear to significantly stimulate differentiation into serotonergic neurons in comparison to the vehicle control, but there was a significant increase between increasing doses (0.01 μg/ml vs 1 μg/ml, $p = 0.0178$).

These observations may be due to serotonergic neurons already being fully differentiated at the time of treatment, as their birthdates are expected to be mostly complete by E15.5 (Pham et al., 1991; Bergner et al., 2014), which coincided with the timeline of ENCDC isolation. Propagation of ENCDCs in culture and subsequent passages using enzymatic dissociations may have further driven the ENCDCs to express a more perinatal timepoint, which was supported by the emergence of dopaminergic neurons in the fifth subculture. Therefore, treatment with these bacterial products might require exceptionally strong stimulation to result in any significance in terms of influencing proportions of serotonergic neurons.

Nitrergic neurons on the other hand constitute a heterogenous mixture of both, early born and late born neurons. Their birthdates typically peak at E15.5, but they are known to continue undergoing terminal differentiation into the early postnatal period (Bergner et al., 2014). Bacterial influences on the development of nitrergic neurons may also be strongest on the late born nitrergic neurons as their fate is still likely plastic.

Finally, dopaminergic neurons are of late born nature. They arise in the perinatal period and continue their terminal differentiation two weeks into the postnatal period (Pham et al., 1991; Bergner et al., 2014). We did not observe any significant effects of

bacterial products on influencing differentiation into dopaminergic neurons, and this could be due to two factors.

The first consideration would be that the treatment of ENCDCs with bacterial products was too early in dopaminergic neuron development to have an effect on their cell fate, and future work analyzing dopaminergic neurons specifically should strive to replicate such experimental conditions in ENCDC cultures that are in later stages of perinatal development.

A second consideration would be that these late born neurons are perhaps not as responsive to microbial products as we observed in early born neurons. It has previously been shown that serotonergic neurons, which arise between E9.5-E15.5 (Pham et al., 1991), may influence the development of perinatal dopaminergic neurons (Li et al., 2004), as mice lacking tryptophan hydroxylase 2 (TPH2) expression, an enzyme involved in serotonin synthesis, exhibited a reduction in dopaminergic populations (Li et al., 2011). Additionally, Li and colleagues (2011) found that ENCDC cell cultures exhibited 5-HT dependent increases in dopaminergic neurons in culture as well as increases in total neurons, suggesting that the effects of 5-HT on promoting enteric neuron survival and development are not restricted to dopaminergic neurons (Li et al, 2011). Indeed, 5-HT may also have an effect on other late born neurons such as GABAergic, CGRP-expressing, and even a subset of nitrergic neurons born after E15.5 (Li et al., 2011).

Taken together, these results may indicate that microbial products exert the most effect on early born neurons of serotonergic and nitrergic subtypes. These early interactions and the subsequent evolution of these early born neurons may provide the basic framework

for the developing ENS. Once established, these early born neurons may affect development of late born neurons through input from neurotransmitter. How these neurotransmitters might influence differentiation into one subtype of neuron over another remains unclear.

4.1.3 Effects of Bacterial Products on Apoptosis of Cultured ENCDCs

We demonstrated a notable lack of significance in apoptosis across all bacterial product conditions and concentrations. These findings are consisted with the notion that apoptosis is not an important characteristic of ENCDC maturation and ENS development.

Programmed cell death is an important hallmark of nervous system development and maturation (Enomoto, 2009). This characteristic is present in a wide variety of nervous system components, from the superior cervical ganglion, to the retinal ganglia, and even in the dorsal root ganglia (Kristiansen & Ham, 2014; Strom & Williams, 1998; Bennett et al., 2002). Previous research suggests that an important driving force for the abundance of nerve cell death in development may be due to competition for target-derived trophic factors, of which nerve growth factor is the most well studied type (Kristiansen & Ham, 2014; Greene et al., 2007).

A recent publication has demonstrated for the first time that the adult enteric nervous system does undergo apoptosis akin to other neurons. Kulkarni and colleagues (2017) showed that approximately a tenth of all myenteric neurons are tagged at all times for caspase-3 cleavage, and that roughly a third of these cells undergoes apoptosis within 7 days, or equal to approximately 50% cell death per day of caspase-3 labelled neurons

(Kulkarni et al., 2017). This is consistent with previous findings in other neurons types by Kristiansen and Ham (2014) who showed that there was 50% cell death of neurons from the superior cervical ganglion within 24 hours of deprivation of nerve growth factor (Kristiansen & Ham, 2014; Kulkarni et al., 2017). Prior to these findings, it was believed that myenteric neurons do not undergo apoptosis and are largely static until factors such as onset of disease result in cell death (Kulkarni et al., 2017).

In contrast, no evidence to date has been found on notable apoptosis in ENCDCs specifically. A study by Gianino and colleagues (2003), showed a marked lack of apoptosis at developmental stages from E12.5 to adult mouse myenteric plexus wholemount preparations when stained for cleaved caspase-3 (Gianino et al., 2003). Another study by Chalazonitis and colleagues (2001) demonstrated a mild response to neurotrophin-3 depletion on cultured ENCDCs which left the cultures undisturbed in terms of the proportion of viable neurons; furthermore, the limited population of cells that did respond to the neurotrophin-3 withdrawal by undergoing apoptosis were found to be a subset of neurons that selectively express the corresponding receptor, tropomyosin receptor kinase C (TrkC), and not necessarily the ENCDCs that provide much of the framework for the developing ENS (Chalazonitis et al., 2001). Interestingly, apoptosis of ENS precursors was detected in vagal neural crest cells as they migrate towards the foregut and prior to foregut invasion (Wallace et al., 2009). Taken together, these most recent findings, while still controversial, seem to indicate that apoptosis is not a typical characteristic of ENCDCs during ENS development; however, apoptosis may be a normal process in ENCDC precursors migrating from the vagal neural crest as well as adult myenteric neurons, as is

typical of other nerves within the nervous system. The conclusions drawn from these findings provide an explanation for the lack of significance observed in our project, where no significant differences were seen in apoptosis of cultured ENCDCs irrespective of bacterial product concentration or type.

4.2 Limitations

There are several limitations in the experiments performed in this project. Primarily, *in vitro* models are limited in the extent of conditions found *in vivo*, such as mimicking of the mesenchymal environment and influences from the immune system and hormones. Furthermore, isolated cells may differ in their gene expression compared to cells in native environments. Zellmer and colleagues (2010), showed that primary hepatocytes upregulate or downregulate hundreds of genes once isolated (Zellmer et al., 2010). Therefore, results obtained under culture conditions may not be representative of *in vivo* activity.

We were unable to obtain duplicate conditions for a subset of bacterial conditions. The TUNEL flagellin data were obtained as singlets. The vehicle controls were also obtained as singlets, as was the chemical coding of subculture 4. This prevented us from obtaining a better representation of direct bacterial product influences on ENCDCs by reducing confounding variables such as inconsistent concentrations during aliquoting.

Controls to assess bacterial product purity were not performed here. While bacterial products were carefully chosen based on reported purities, the potential for cross-reactivity with other receptors and pathways cannot be excluded without incorporating proper controls. As a result, we acknowledge the limitations to our claims presented here referring

to direct effects of specific bacterial products on ENCDC fate. Future experiments should assess product purity and ensure consistent increases in concentrations using reporter cell lines. Such experiments may entail testing LPS specificity using human embryonic kidney cells 293 (HEK)-Blue TLR4 and HEK-Blue TLR5 platforms. One would expect increased detection of secreted embryonic alkaline phosphatase (SEAP) in HEK-Blue TLR4 cultures, but not HEK-Blue TLR5 cultures. Stimulation of SEAP in HEK-Blue TLR5 cultures with LPS would indicate impurities in LPS stock and cross-reactivity with other pathways. Similar controls could be applied across all bacterial product conditions.

The cultures produced in this project were of C57Bl/6 origin. Increasing research is shedding light on the important role of genetics in driving strain-specific differences of commonly used laboratory mice (Marques et al., 2011). This may indicate that the effects of bacterial products on ENCDC fate observed in C57Bl/6 mice here may not be consistent in other strains.

A germ free ENCDC model may provide a clearer signal for assessing the true role of bacterial products on directing ENS development. While the first largest colonization of gut bacteria occurs during parturition and infancy, it is now recognized that the intrauterine environment is not sterile (Jimenez et al., 2008). This was first identified by the presence of *Enterococcus*, *Streptococcus*, *Staphylococcus*, or *Propionibacterium* isolated from human embryonic cord blood (Jimenez et al., 2005). Later research showed newborn meconium samples to be dominated by bacteria of intrauterine origin such as *Escherichia*, *Shigella*, *Leuconostoc*, *Enterococcus*, and *Lactococcus*; that is, they differed in composition from microbial communities typically found in the maternal vagina

(*Lactobacillus*), skin (*Corynebacterium, Propionibacterium*), or feces (*Bacteroides, Clostridium*) (Gosalbes et al., 2013). The presence of such bacteria in the intrauterine environment and their colonization of the fetal GI tract is believed to be associated with priming of the fetal gut and immune system via the gut-associate lymphoid tissue (GALT) (Jimenez et al., 2005). These findings suggest not only the presence of bacteria in the fetal gut but also its importance in normal development. This may indicate that the ENCDCs isolated here have already been exposed to a limited number of bacterial products *in utero*, making them no longer entirely naïve in development.

The concentration of $p75^{NTR+}$ antibody used for flow cytometry was high (1:10). Titration of the antibody resulted in a drastic decrease in positive cells. One consideration for such results could come from omitting sodium azide from the flow cytometry staining protocol. Sodium azide acts as a metabolic inhibitor and has been shown to prevent surface antigen internalization in an ATP-dependent manner during processing, thereby preventing loss of fluorescence intensity (Sato et al., 2009; Harvey et al., 1999). Future repeats of this experiment should consider achieving optimal antibody concentrations to reduce nonspecific binding with the use of sodium azide as a means of reducing confounders such as internalization of cell surface markers.

The second consideration could be that the dissociation of cell cultures using Accutase immediately before processing for flow cytometry may have resulted in receptor cleavage. For the present study, Accutase was chosen due to its significantly gentler dissociation of adherent cells and significantly increased cell viability upon dissociation as compared to trypsin or collagenase (Li et al., 2015; Bajpai et al., 2008). Previously

published comparisons between culture characteristics of Accutase dissociated cultures and collagenase dissociated cultures demonstrated a larger proportion of undifferentiated cells with Accutase treatment, which was a particularly important objective in the study presented here (Bajpai et al., 2008). Previous research also showed that dissociation by Accutase yielded significantly higher initial viability of neural stem cells as well as cell survival (90-95% vs 70-80%) (Wachs et al., 2003). Furthermore, Accutase treatment yielded faster formation and an increased quantity of neurosphere aggregates as compared to trypsin (Wachs et al., 2003). In fact, Accutase was specifically formulated for the purpose of flow cytometry; that is, to reduce cleavage of important cell surface antigens. Despite this, Accutase is known to exhibit proteolytic and collagenlytic activity. The exact formulation of Accutase remains propriety and so its exact effects on cell surface markers remain unconfirmed (Wachs et al., 2003). One study investigated the impact of various methodologies of macrophage detachment and found both, trypsin and Accutase, caused cleavage of important cell surface markers (Chen et al., 2015). Future work on this project should look into alternative methods of cell dissociation that do not involve the use of enzymatic activity, such as cell scraping and manual trituration. As ENCDCs are known for their propensity to naturally form neurosphere aggregates in culture (Nishikawa et al., 2015; Hotta et al., 2013), single-cell dissociation can further be ensured by straining through a cell strainer prior to processing for flow cytometry.

Despite such high antibody concentrations for flow cytometry, we were able to validate that our cell population was in fact of neuronal origin using immunofluorescence. We were further able to confirm that these were our cells of interest based on our

experimental findings in incubations with bacterial products, where significant differences were found in proliferation and differentiation using different bacterial products. Our one constant was the consistent lack of significance across the various conditions related to apoptosis. The lack of apoptosis is a unique characteristic of ENCDC development and these findings provide additional support that our cultures were likely highly pure ENCDC cultures.

4.3 Future Directions

In accordance with previous research which demonstrated that early born enteric neurons influence development of late born enteric neurons (Li et al., 2004), our findings appear to suggest that ENCDC exposure to microbial products in prenatal ENS development may have a stronger role in influencing programming of early born enteric neurons. We hypothesize that once this framework of early enteric neurons is established, microbial products no longer play as important a role in the terminal differentiation of late born enteric neurons as does neurotransmitter input from the already established early neurons. Further validation of our findings is required through the involvement of a broader panel of late born enteric neurons, such as CGRP neurons, to ensure that these findings are consistent. Additionally, the exact mechanisms underlying early born neuron influence on the development of late born neurons remain unknown.

Future work in this area may also involve exploring the expression of appropriate receptors that correspond to these microbial products, and whether there is an upregulation

of these receptors that corresponds to bacterial product dose. Activation of appropriate pathways should also be assessed and determined if they act in a dose-dependent manner.

Finally, a study by Lindley and colleagues (2008) demonstrated the ability to transplant cultured enteric progenitor neurospheres of neonatal human gut origin into aganglionic gut segments of mice. They demonstrated the rescuing of muscle contractile frequencies to lower normal limits of a ganglionic gut. A similar research approach can be taken in a future experiment, where cultured ENCDCs are transplanted into aganglionic gut segments of mice, along with supplementation of common bacterial products either through gavage feeding or enema administrations to test whether these bacterial products could further improve the differentiation and innervation of the deficient ENS. It is likely that these bacterial products would induce a concurrent inflammatory response; therefore, careful titration of bacterial products would be imperative.

4.4 Conclusions

Here, we demonstrated the successful establishment of an *in vitro* model of ENCDC cultures that were highly pure and viable. Cultures were characterized for chemical coding to determine the length of time that the cells could remain in culture in a relatively undifferentiated state. We showed the maintenance of ENCDCs in a highly proliferative and immature state for four subcultures after isolation.

Using the *in vitro* model, we characterized cultures after incubation with common bacterial products to determine direct influences on the developmental fate of ENCDCs. Our results are suggestive of bacterial product influences on ENCDC proliferation and

differentiation into serotonergic and nitrergic neurons. We were not able to show an influence of bacterial products on ENCDC apoptosis or differentiation into dopaminergic neurons under any conditions.

These results may indicate that the microbiome plays an important role on perinatal development of the ENS. Future work is required to validate these results and determine the mechanisms by which such interactions occur.

APPENDIX

Table 1. Media components and volumes. ENCDC isolation required several variants of NSM media during processing (processing media, NSM antibody wash). This table also represents NSM+ plating media, which was used immediately post-ENCDC isolation to facilitate cell adherence. This media was changed after 24 hours to the NSM+ maintenance media, which was used for culture maintenance and growth until cryopreservation or processing for immunofluorescence.

MEDIA				
Component	**Processing Media**	**NSM Antibody Wash**	**NSM+ Plating Media**	**NSM+ Maintenance Media**
DMEM/F12 (Sigma D8437-500ml)	97.5%	95.59%	75.89%	95.89%
Heparin (Sigma H3149-10KU)	--	0.01%	0.01%	0.01%
bFGF (Sigma F0291-25UG)	--	--	0.08%	0.08%
EGF (Invitrogen PMG8041)	--	--	0.02%	0.02%
B27 (Invitrogen 17504044)	--	2%	2%	2%
N2 (Invitrogen 17502048)	--	1%	1%	1%
Penicillin/streptomycin (Invitrogen 15140122)	1%	1%	1%	1%
Horse serum (Invitrogen 26050088)	1%	--	20%	--
GDNF	0.5%	--	--	--
BSA	--	250 mg	--	--
EDTA (0.5 M stock) (Sigma EDS)	--	0.4%	--	--

Table 2. ENCDC yield and viability at every passage for 5 subcultures. Represented are the subculture number, date of cell passage, number of cells after dissociation, and percent viability. All cells were not reseeded for establishment of subsequent subcultures. *Assessment of culture purity for ENCDC marker, $p75^{NTR+}$, and viability was done on subcultures 3 and 4. ⁑$5x20^7$ cells from subculture 4 were cryopreserved for future use. †Characterization of culture chemical coding and proliferation was performed on subcultures 4 and 5. ‡Exposure to bacterial products was done on subculture 5.

Subculture	# Cells	% Viability	# cells re-seeded
0	2,212,500	99.4%	2,212,500
1	7,175,000	100%	7,175,000
2	34,350,000	100%	34,350,000
3*	106,250,000	100%	--
4*⁑†	75,500,000	100%	12,300,000
5†‡	148,000, 000	100%	--

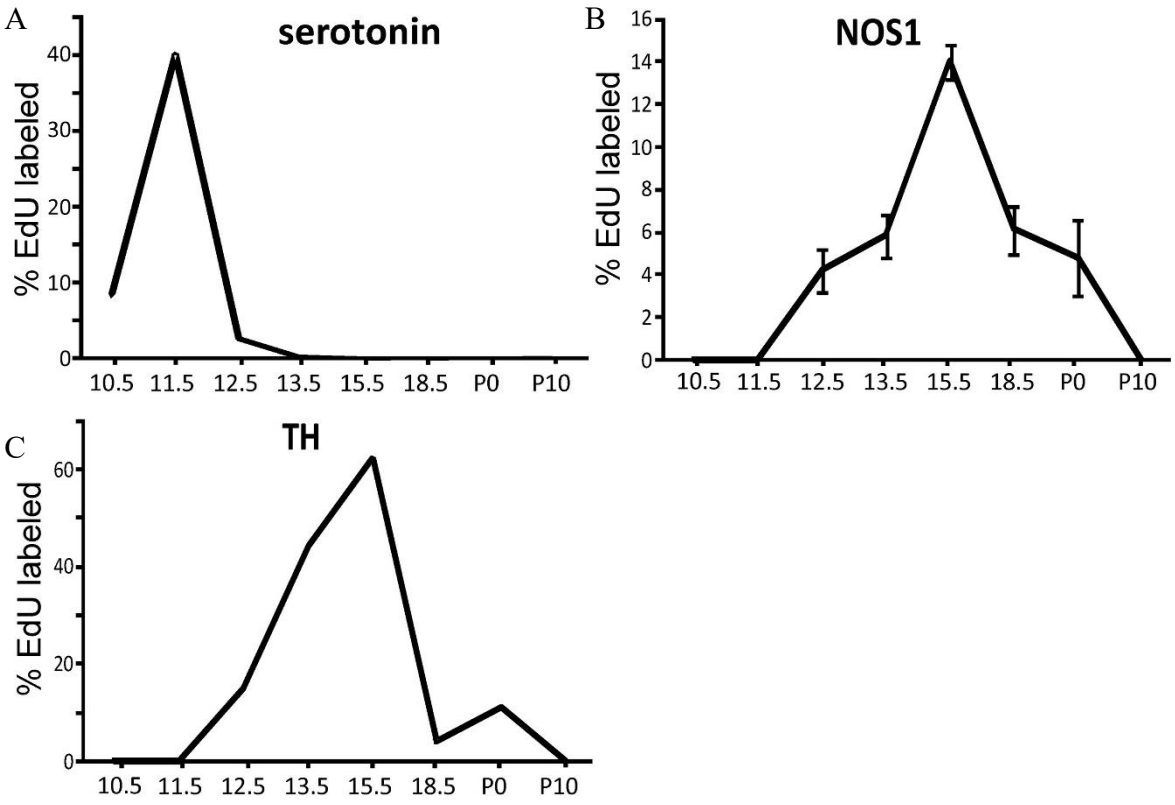


Figure 1. Birthdates of myenteric neuron subtypes. Percentage of myenteric neuron subtypes retaining EdU staining at different developmental stages. (A) Serotonergic neurons exhibit cell cycle exits from before E10.5 to E13.5, with a peak birthdate at E11.5. (B) Nitrergic (NOS1) neurons have birthdates between E11.5-P10, with a cell cycle exit peak at E15.5. (C) Dopaminergic (TH) neurons exhibit cell cycle exits between E11.5-P10, with a peak time of cell cycle exit at E15.5. Figure adapted from Bergner et al., 2014.

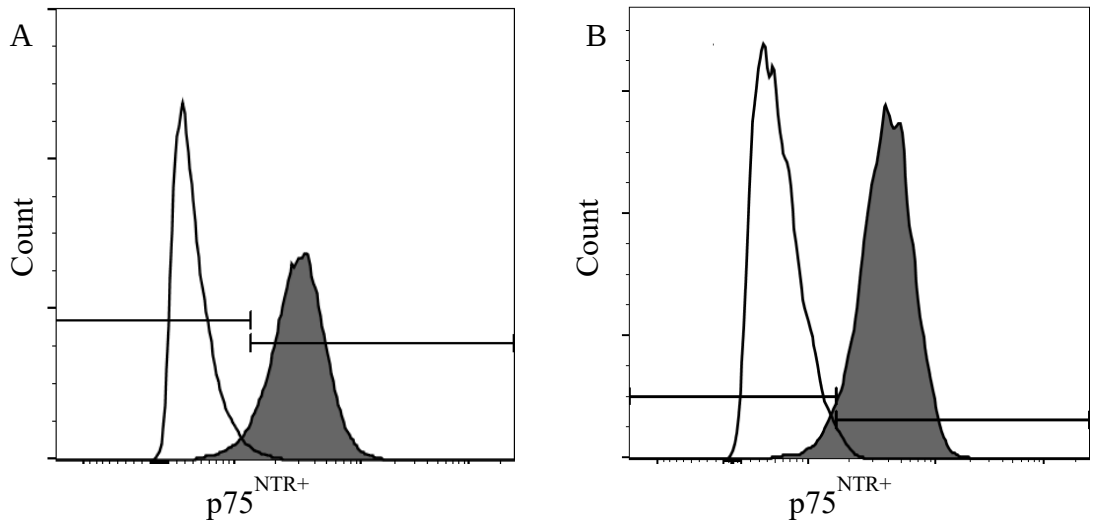
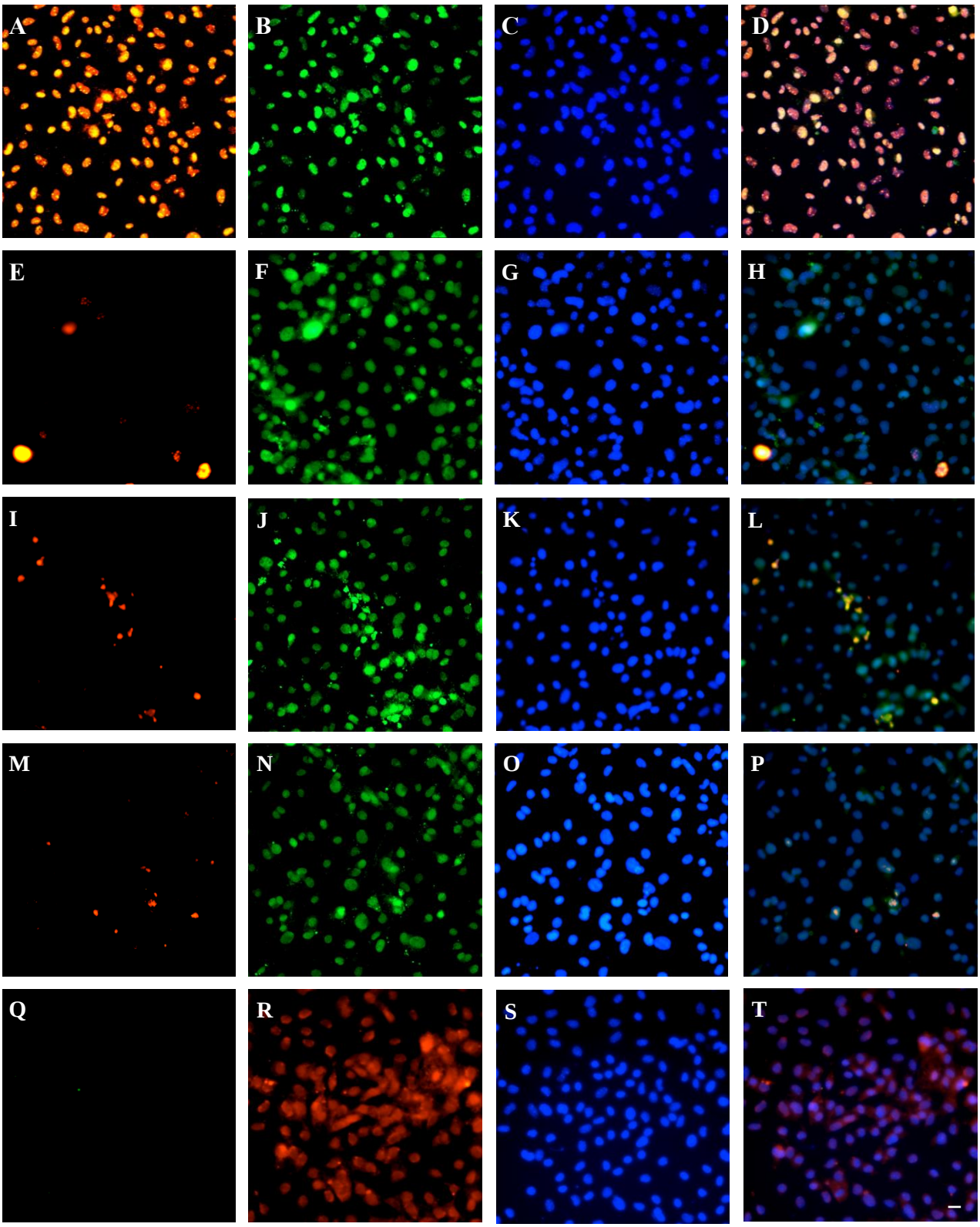


Figure 2. Flow cytometry assessment of ENCDC cultures. Target cells were stained with antibodies against immature neuronal marker, $p75^{NTR+}$-FITC. Proportion of $p75^{NTR+}$ expressing cells are depicted by the gray peak for subculture 3 (A, 95.1% $p75^{NTR+}$-positive, 97.3% viability) and subculture 4 (B, 96.1% $p75^{NTR+}$-positive, 98.2% viability).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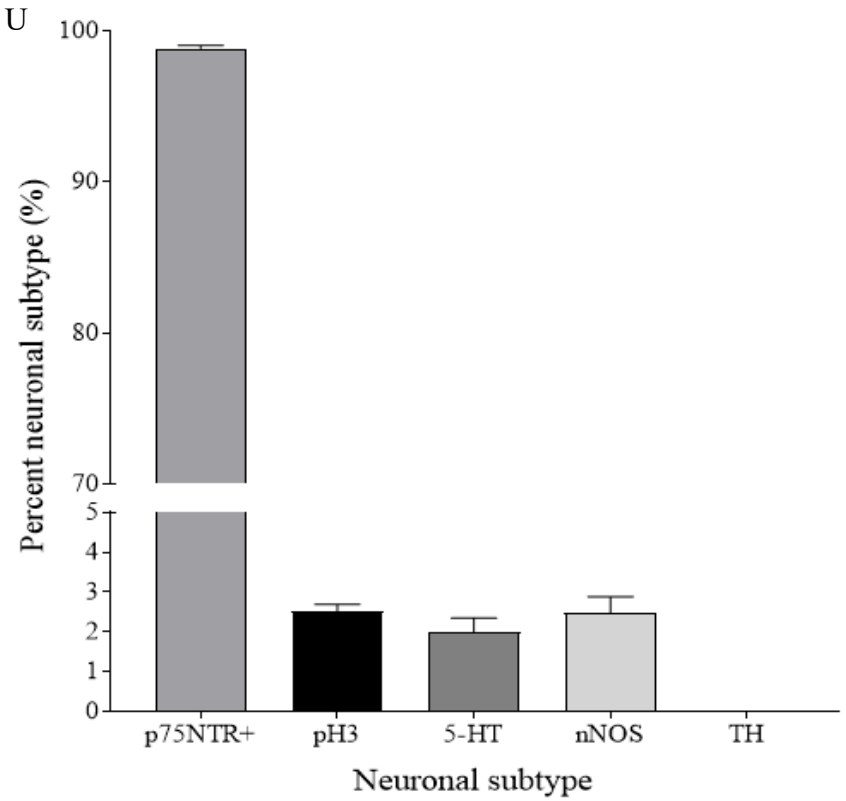

Figure 3. ENCDC culture characteristics of the fourth subculture. (A-D) Proportion of undifferentiated ENCDC (A, $p75^{NTR+}$, red; B, HuC/D, green; C, bisbenzimide, blue; D, overlay of all three channels). (E-H) Proportion of proliferating cells (E, phospho-histone H3, red; F, HuC/D, green; G, bisbenzimide, blue; H, overlay of all three channels). (I-L) Proportion of serotonergic neurons (I, 5-HT, red; J, HuC/D, green; K, bisbenzimide, blue; L, overlay of all three channels). (M-P) Proportion of nitrergic nNOS (M, nNOS, red; N, HuC/D, green; O, bisbenzimide, blue; P, overlay of all three channels). (Q-T) Proportion of dopaminergic neurons (Q, HuC/D, red; R, tyrosine hydroxylase, green; S, bisbenzimide, blue; T, overlay of all three channels). (U) Quantification of neuronal subtypes (% positive cells). Scale bar = 20 µm. Values are present as mean ± S.E.M.

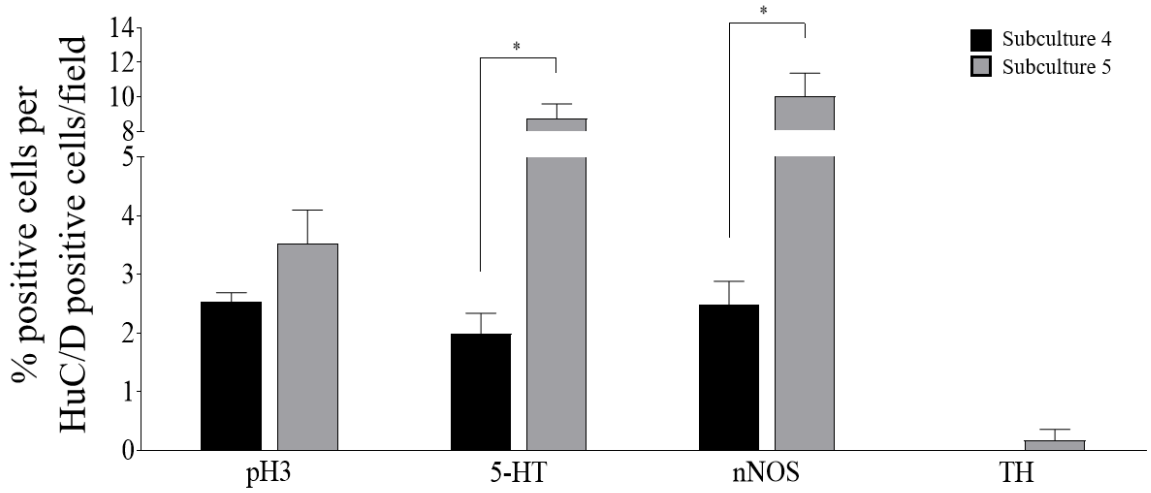

Figure 4. ENCDCs continue to differentiate in culture. There was a significant increase in proportion of serotonergic neurons and nitrergic neurons in subculture 5 (gray) compared to subculture 4 (black). There was no significant difference in proliferating cells or dopaminergic neurons across the cultures. *$p \leq 0.05$. Values are present as mean ± S.E.M.

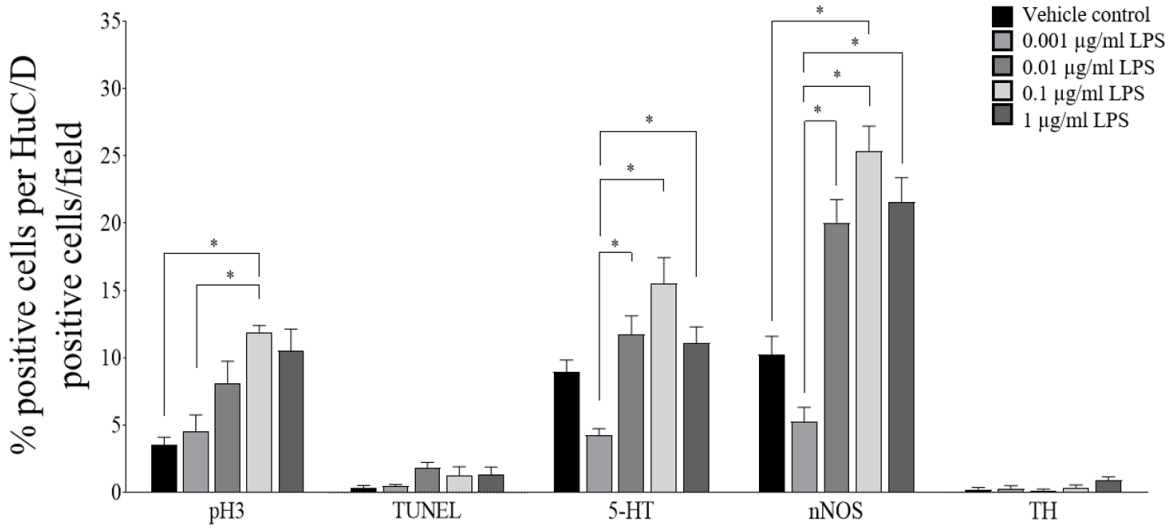

Figure 5. Role of LPS on ENCDC programming. LPS has a significant effect on increasing ENCDC proliferation, and differentiation into nitrergic neurons. LPS does not have a significant effect on ENCDC apoptosis or differentiation into dopaminergic neurons. There was an increase in serotonergic neurons, but this was not significant with the control. *$p \leq 0.05$. Values are present as mean ± S.E.M.

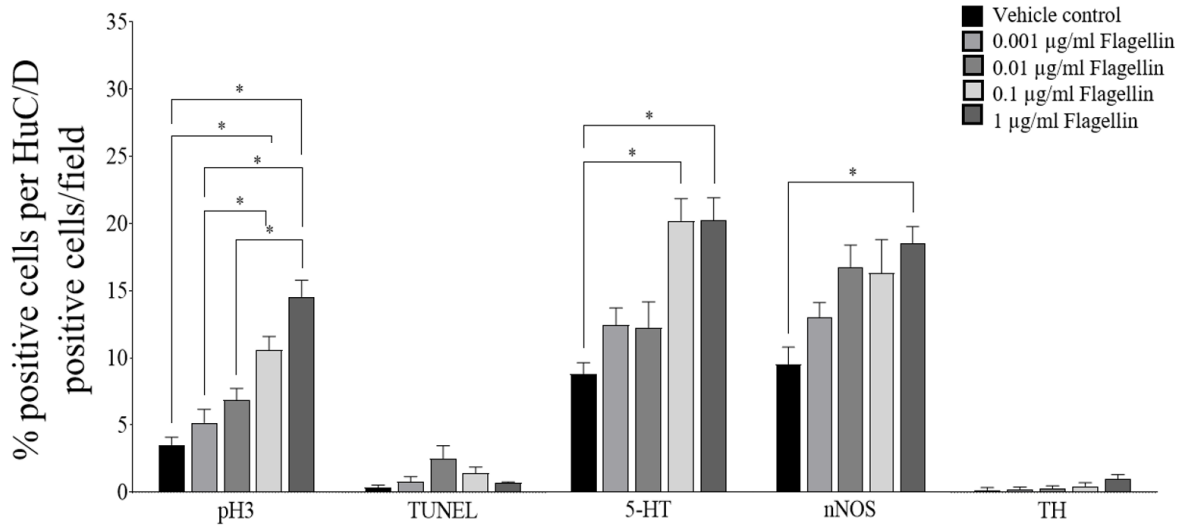

Figure 6. Role of flagellin on ENCDC programming. Flagellin has a significant effect on increasing ENCDC proliferation, and differentiation into serotonergic and nitrergic neurons. Proliferation appears to be dose-dependent. Flagellin does not have a significant effect on ENCDC apoptosis or differentiation into dopaminergic neurons. *$p \leq 0.05$. Values are present as mean ± S.E.M.

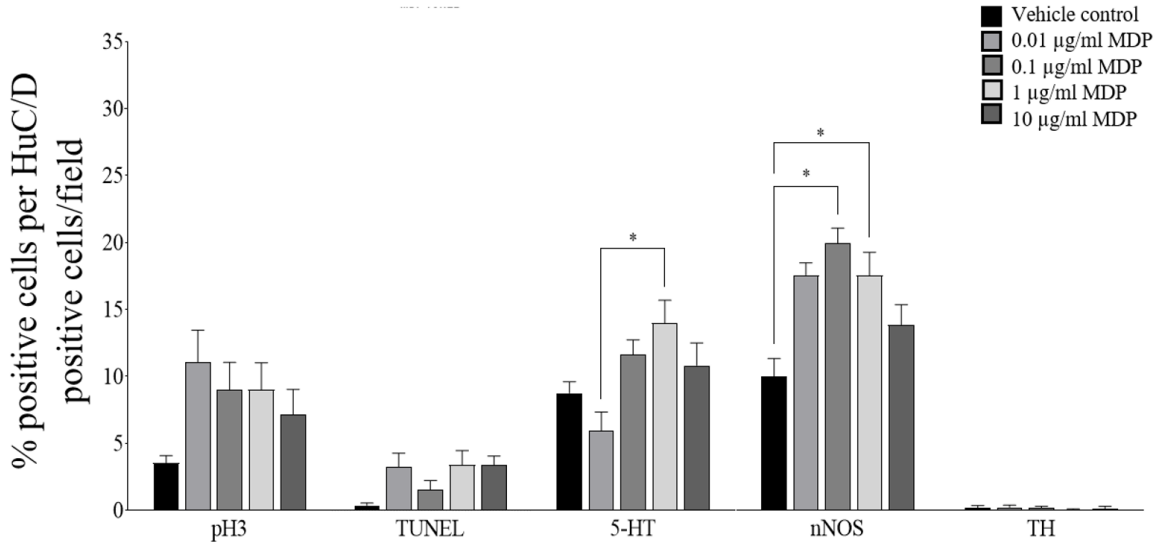

Figure 7. Role of MDP on ENCDC programming. MDP has a significant effect on increasing ENCDC differentiation into nitrergic neurons. MDP does not have a significant effect on ENCDC proliferation, apoptosis or differentiation into dopaminergic neurons. Differentiation into serotonergic neurons was only significant between different doses. *p ≤ 0.05. Values are present as mean ± S.E.M.

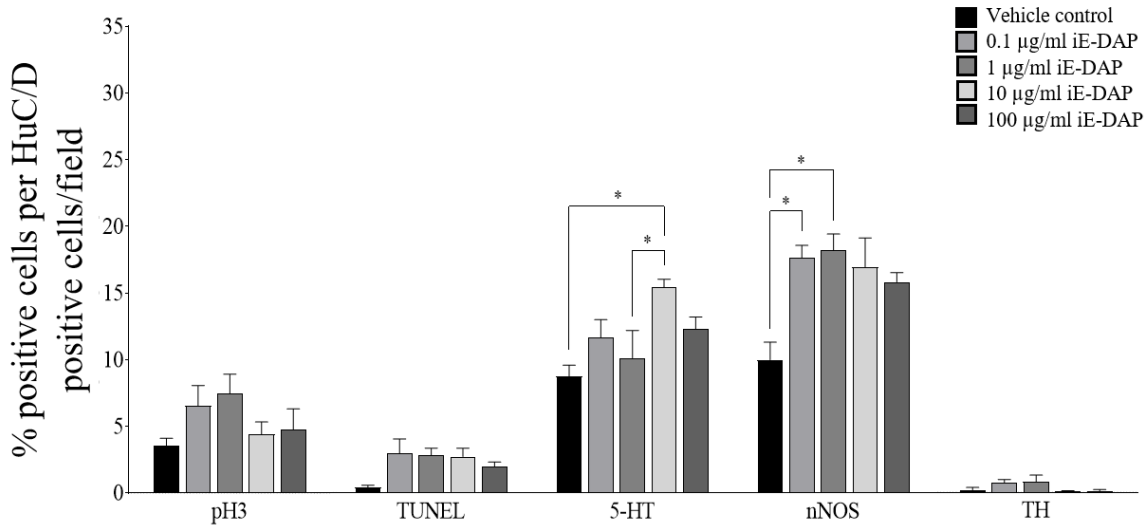

Figure 8. Role of iE-DAP on ENCDC programming. iE-DAP has a significant effect on increasing ENCDC differentiation into serotonergic and nitrergic neurons. iE-DAP does not have a significant effect on ENCDC proliferation, apoptosis or differentiation into dopaminergic neurons. *$p \leq 0.05$. Values are present as mean ± S.E.M.

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