**Immunohistochemical Localization of the Melanocortin-4 Receptor (MC4R) in the Colon Myenteric Plexus**

by

Lauren R. Parker

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 9, 2010

Approved by

Mahmoud M. Mansour, Co-Chair, Associate Professor of Anatomy
Edward E. Morrison, Co-Chair, Professor of Anatomy
John C. Dennis, Research Fellow IV

Abstract

Alpha-melanocyte stimulating hormone (α-MSH) is a tricdecaneuropeptide that can bind four melanocortin receptors (MC1R, MC3R-MC5R) to exert various receptor-dependent effects. MC4R regulates several functions which include energy homeostasis, enhancement of sexual function, blood pressure, and heart rate, and suppression of inflammation. Although MC4R is predominantly expressed in the central nervous system (CNS), peripheral expression was also observed in the enteric nervous system (ENS) of the stomach and duodenum. α-MSH exerts anti-inflammatory effects via MC4R in the brain and attenuates experimental-induced inflammatory bowel disease (IBD) in the colon by an unknown mechanism. We asked if ENS MC4R expression extends to the colon and if treatment with Melanotan II (MTII), an α-MSH analog, or leptin, stimulates increased MC4R expression. We used reverse-transcriptase polymerase chain reaction (RT-PCR) to detect MC4R mRNA in the rat colon and immunohistochemistry (IHC) to determine cell types that express MC4R. We focused on enteric glial cells and myenteric neurons using floating sections of longitudinal muscle- myenteric plexus (LMMP) and formalin-fixed, paraffin-embedded rat colon cross-sections. RT- PCR showed MC4R mRNA expression in the colon and IHC localized MC4R to the myenteric plexus. Likewise, a co-localization assay showed that the MC4R-positive cells were also positive for neuron-specific beta III tubulin. In enteric glial cells, MC4R was co-localized with

glial fibrillary acidic protein (GFAP), a glial cell marker. There was no significant difference in MC4R expression among MTII, leptin, or vehicle treatment groups.

## Acknowledgements

I would like to thank my major professor, Dr. Mansour, as well as my committee members Dr. Dennis and Dr. Morrison for their support and guidance throughout this process and for this opportunity. I have learned a tremendous amount under their direction.

I would also like to express gratitude to my parents, Lawrence and Shirleen Parker, for their love and encouragement and for always believing in me. They have been by my side at every major milestone and accomplishment in my life, including this one.

I want to acknowledge Mrs. Karen Wolfe, who has been an invaluable resource and a good friend, and Ms. Amanda Hazi for her diligence and enthusiasm in the laboratory. I would like to thank the faculty and staff of the Department of Anatomy, Physiology, and Pharmacology for making it such an inviting and pleasant learning and work environment. Lastly, I want to acknowledge fellow graduate students India D. Napier, Manjunatha K. Nanjappa, Victoria A. Light, and Martha Washington for all of their assistance and advice.

Table of Contents

Abstract ..... ii

Acknowledgements ..... iv

List of Tables ..... vii

List of Figures ..... viii

List of Abbreviations ..... x

Introduction ..... 1

Literature Review ..... 2

Melanocortin System/Receptors ..... 2

Central and Peripheral Expression of MC4R ..... 5

Role of Leptin in the Regulation of the Melanocortin System ..... 5

Anatomy of the Colon ..... 7

Enteric Nervous System (ENS) ..... 8

Enteric Glial Cells ..... 13

Expression and Potential Role of MC4R in the Gastrointestinal Tract ..... 16

Inflammation ..... 17

Inflammatory Bowel Disease (IBD) in the Colon ..... 18

Role of Luminal Flora in IBD ..... 20

Role of Intestinal Epithelium in IBD ..... 21

Inflammation and Enteric Neuropathy.....22

Current therapies for IBD .....26

α-MSH and inflammation .....28

Objectives .....38

Materials and Methods.....40

Results.....48

Discussion.....61

References.....65

List of Tables

Table 1: Melanocortin Receptor Location, Ligand(s), and Function.....33

Table 2: Primary Antibodies used for Immunohistochemistry.....34

Table 3: Secondary Antibodies used for Immunohistochemistry.....34

List of Figures

Fig. 1: Post-translational Modification of POMC Yields Melanocortin Peptides.....35

Fig. 2: Leptin Modulates the Melanocortin System.....36

Fig. 3: Layers of the Wall of the Colon .....37

Fig. 4: H&E Stained Paraffin-Embedded Rat Colon Cross-Section.....51

Fig. 5: Immunofluorescent Labeling of Myenteric Neurons and Enteric Glial Cells.....52

Fig. 6: GFAP is a Marker for Enteric Glial Cells in the Colon and Astrocytes in the Brain.....53

Fig. 7: Semi-quantitative RT-PCR Detection of MC4R in the Rat Gastrointestinal Tract and Hypothalamus .....54

Fig 8: Semi-quantitative RT-PCR Detection of MCR Subtypes -1, -3, -4, and -5 in the Rat Colon .....55

Fig. 9: Immunohistochemical Localization of MC4R in the Colon Myenteric Plexus.....56

Fig. 10: Co-localization of Beta III Tubulin and MC4R in the Colon Myenteric Plexus.....57

Fig. 11: Co-localization of MC4R and GFAP in Colon Enteric Glial Cells .....58

Fig. 12: Immunohistochemical Comparison of MC4R Expression among MTII, Leptin, and Vehicle Groups.....59

Fig. 13: Quantitative RT-PCR Analysis of Treated and Control ICV Groups.....60

List of Abbreviations

| | |
|---|---|
| ACTH | Adrenocorticotropic Hormone |
| AgRP | Agouti-related Protein |
| α-MSH | Alpha-Melanocyte Stimulating Hormone |
| β-MSH | Beta-Melanocyte Stimulating Hormone |
| CNS | Central Nervous System |
| cAMP | Cyclic AMP |
| DMV | Dorsal Motor Nucleus of the Vagus Nerve |
| EGCs | Enteric Glial Cells |
| ENS | Enteric Nervous System |
| γ-MSH | Gamma-Melanocyte Stimulating Hormone |
| GFAP | Glial Fibrillary Acidic Protein |
| H&E | Hematoxylin & Eosin |
| IHC | Immunohistochemistry |
| iNOS | Inducible Nitric Oxide Synthase |

| | |
|---|---|
| IBD | Inflammatory Bowel Disease |
| IFN-γ | Interferon-Gamma |
| IL- | Interleukin- |
| icv | Intracerebroventricular |
| ip | Intraperitoneal |
| MC(1-5)R | Melanocortin (1-5) Receptor |
| MTII | Melanotan II |
| NO | Nitric Oxide |
| NF-κB | Nuclear Factor Kappa B |
| PANS | Parasympathetic Branch of the Autonomous Nervous System |
| PVN | Paraventricular Nucleus of the Hypothlamus |
| PNS | Peripheral Nervous System |
| POMC | Pro-opiomelanocortin |
| RT-PCR | Reverse-Transcriptase PCR |
| SANS | Sympathetic Branch of the Autonomic Nervous System |
| TNF-α | Tumor Necrosis Factor-alpha |
| VIP | Vasoactive Intestinal Polypeptide |

## Introduction

The majority of studies on MC4R predominantly address MC4R expression in the CNS and its role in energy homeostasis and obesity. However, researchers have also discovered several other peripheral MC4R distribution sites and functions that are not related to obesity and/or energy homeostasis. For example, MC4R has been localized in the ENS, specifically to the myenteric plexus of the duodenum and stomach.[1] Aside from energy homeostasis, MC4R has also been shown to modulate cardiovascular and sexual function and suppress inflammation. [2, 3] Additionally, central administration of α-MSH has been shown to suppress fever, while α-MSH intensifies fever in the presence of an MC4R antagonist. [3]

Because the colon is subject to inflammatory processes and is the primary site for inflammatory bowel disease (IBD), the objectives of this study are to 1) determine whether MC4R is expressed in the rat colon using semi-quantitative RT-PCR, 2) identify specific cell types in the colon that express MC4R with emphasis on myenteric neurons and enteric glial cells (EGCs), and 3) determine whether MC4R is functional in these locations by observing the effect of central and peripheral administration of MTII on MC4R expression.

## Literature Review

### Melanocortin System/Receptors

Melanocortins are biologically active neuropeptides with neuromodulatory actions that are formed by post-translational processing of pro-opiomelanocortin (POMC) and share a seven amino acid core sequence. [3, 4] Neuropeptides are defined as peptides that serve as intercellular messengers after being released from the nervous system. [5] POMC is selectively cleaved by prohormone convertases into alpha, beta, and gamma melanocyte-stimulating hormones (α-,β-, and γ-MSH) and adrenocorticotropic hormone (ACTH) (Fig. 1). [4] The tissue-specific activity of the prohormone convertases determines which hormones are expressed where.[3] POMC is processed in the anterior pituitary to yield ACTH, which stimulates the adrenal glands to secrete adrenal steroids. [4] To form the tridecapeptide hormone α-MSH, POMC is first cleaved into ACTH and β-lipotropin by prehormone convertase 1 which is then followed by further cleavage of ACTH into α-MSH by prehormone convertase 2. [3] β-MSH is processed from β-lipotropin and γ-MSH is derived from the N-terminal of POMC. Synthesis of α- and γ-MSH occurs in the intermediate lobe of the pituitary. [4]

Melanocortins induce their effects by binding to melanocortin receptors expressed on target cells. [6] There are five melanocortin receptor subtypes that have been identified

(MC1R-MC5R) and named based on the order in which they were cloned. [2] Melanocortin receptors are seven-transmembrane G-protein- coupled receptors (GPCRs). MC1R is expressed in melanocytes and is responsible for skin pigmentation. [2] Stimulation of melanogenesis, which increases skin pigmentation, was the first known function of α-MSH. MC1R is also found in immune cells (neutrophils, monocytes, dendritic cells, endothelial cells, and B lymphocytes), glioma cells, and astrocytes. [3] It binds α- and β-MSH and ACTH with equal affinity, and binds γ-MSH with less affinity. MC2R is expressed in the adrenal cortex. [3] It selectively binds ACTH to stimulate adrenocortical steriodogenesis.[3, 6] MC3R is expressed in the CNS, gastrointestinal tract, and kidney and plays a role in energy homeostasis, feeding, and natriuresis.[3] Areas in the CNS in which MC3R is most prominent include the ventromedial hypothalamus and ventral segmental and raphe areas. MC3R binds α-, and β-, and γ-MSH and ACTH with equal affinity. [2]

MC4R is the predominant MCR subtype in the CNS, expressed in almost all of the major brain regions including the cerebral cortex, hypothalamus, thalamus, brainstem, and spinal cord. [4] It regulates energy homeostasis and enhances erectile function in males and sexual desire in females.[2] Intracerebroventricular (icv) doses of synthetic MC4R agonists increase blood pressure and heart rate in wild-type but not in MC4R-deficient mice. [7]

In the CNS, MC4R is most prominently expressed in the paraventricular nucleus of the hypothalamus and the dorsal motor nucleus of the vagus nerve. [3] Its binds α-, β-, and γ-MSH and ACTH, with highest affinity for β-MSH.[8] The major focus of MC4R research has been on obesity. Multiple studies have shown its importance in energy homeostasis.[3] One study demonstrated that MC4R knockout mice (-/-) were severely

obese, while heterozygous mice (+/-) had milder obesity. Another study showed that MC4R (-/-) mice had a diminished response to the anorexigenic effects of the MC4R agonist MTII. [9, 10] MC4R is also a mediator of attenuation of inflammation in the brain.[3]

MC5R is integral in the synthesis and secretion of exocrine gland products and is expressed in exocrine cells of sebaceous, Harderian, lacrimal, preputial, and prostate glands.[3, 11] It binds with highest affinity to α-MSH. [3] With all MCRs, ligand stimulation results in activation of adenylate cyclase and an increase in intracellular cyclic AMP (cAMP). MCR signaling has also been linked with other second messenger mechanisms such as inositol triphosphate ($IP_3$)-stimulated increases in intracellular calcium and with mitogen-activated protein (MAP) kinases can also occur.[12, 13]

Endogenous antagonists have been identified for some of the melanocortin receptors. Agouti, which is produced in the skin, binds MC1R and MC4R and Agouti-related protein (AgRP), which is mainly expressed in the arcuate nucleus of the hypothalamus, subthalamic region, and adrenal cortex , binds MC3R and MC4R to stimulate feeding and inhibit energy expenditure.[2, 3, 13] All together, the melanocortin system consists of the melanocortin peptides, five melanocortin receptors (MC1R-MC5R), and the two endogenous antagonists agouti and AgRP. [13] Table 1 summarizes the melanocortin receptors, endogenous ligands, locations, and functions.

Several endogenous stimuli, such as corticotropin-releasing hormone (CRH), pro-inflammatory cytokines like IL-1 and tumor necrosis factor-alpha (TNF-α), and noxious stimuli like UV irradiation and microbes, direct the production of melanocortins.[6] The significance of melanocortins in processes such as lipolysis, food intake, memory, and

inflammatory responses has been demonstrated with knock-out mice models and genetic studies.[3]

## Central and Peripheral Expression of MC4R

As mentioned, the most prominent areas of MC4R expression in the CNS are in the paraventricular nucleus of the hypothalamus and the dorsal motor nucleus of the vagus nerve. [3] It is also expressed in regions of the thalamus, hippocampus, cortex, amygdala, brainstem and spinal cord and is the major MCR subtype in the CNS. [4, 8] MC4R is expressed by both neurons and astrocytes. [8]

Peripherally, MC4R is expressed in several tissues including adipose tissue and pancreatic neurons. [14, 15] MC4R expression has also been shown in the myenteric plexus of the stomach and dudodenum.[1] During the fetal period, it is expressed in the developing heart, lung, kidney, testis, and some muscles, including those involved in respiration.[8] MC4R is also expressed in the pelvic ganglion of rats and the penis of rats and humans, which are all tissues that regulate erectile function.[13]

## Role of Leptin in the Regulation of the Melanocortin System

Leptin is a 16 kDa peptide, encoded by the *ob* gene, that is produced primarily by white adipose tissue and plays an important role in body weight homeostasis, cardiovascular function, and glucose utilization by peripheral tissues.[16, 17] The amount of circulating leptin is proportional to the amount of fat in the body, specifically the amount

of adipose tissue and possibly adipocyte size. Leptin relays to the brain the amount of energy being stored by the body and exerts anorectic effects in the following ways: it promotes decreased appetite and increased energy expenditure by stimulation of sympathetic innervation to various tissues. Knockout-mouse studies of leptin-deficient (*ob/ob*) and leptin-resistant and leptin-deficient (*db/db*) mice show that both groups display the same characteristics: hyperphagia, decreased energy expenditure, type II diabetes with severe insulin resistance, hypothermia and cold intolerance, infertility, and decreased lean body mass. [17]

Studies have shown that leptin may mediate arterial pressure via the CNS with chronic leptin infusion raising it. Leptin also stimulates glucose utilization by peripheral tissues by way of the CNS. One study showed that intracerebroventricular (icv) infusion of leptin reversed streptozotocin (STZ)-induced diabetes-associated hyperglycemia, hyperphagia, and bradycardia.[16]

Leptin suppresses various orexigenic pathways (Neuropeptide Y, AgRP) while stimulating various anorexigenic pathways (POMC system). The POMC pathway seems to be integral in mediating the CNS actions of leptin with regard to appetite and the cardiovascular system. Leptin signaling results in stimulation of POMC neurons in the arcuate nucleus which express leptin receptors and the subsequent release of α-MSH and activation of MC3R/MC4R in various brain nuclei (Fig. 2). [4, 16]

## Anatomy of the Colon

The human large intestine consists of the cecum, the ascending, transverse, sigmoid, and descending colon and the rectum and can be 1.5 to 1.8 meters long. It has a greater average diameter than the small intestine.[18] Water, electrolytes, and vitamins are absorbed in the proximal colon and feces are formed and stored in the distal colon until they are defecated. [18] Mucus secretion and microbial action on the ingesta also take place in the large intestine.[19]

The wall of the colon consists of four layers, from the lumen outward: tunica mucosa, tela submucosa, tunica muscularis, and tunica serosa (Fig. 3). The tunica mucosa lines the lumen and is composed of the epithelial lining, lamina propria, and lamina muscularis. Intestinal glands (or crypts) extend into the lamina muscularis. The tunica mucosa of the colon is significantly thicker than that of the small intestine due to the increased length of these glands.[19] Simple columnar epithelium consisting of surface absorptive cells, diffuse neuroendocrine system cells, and goblet cells (more abundant than in the small intestine) make up the epithelial layer.[19, 20] The lamina propria is characterized by loose connective tissue that surrounds the intestinal glands. Unlike the small intestine, the colon lacks villi, making the mucosal surface smooth. The lamina muscularis consists of interrupted inner circular and outer longitudinal layers of smooth muscle from distention of the submucosa by lymphatic tissue. [19]

The tela submuscosa is the second layer of the colon wall and it is made up of loose-connective tissue with submucosal glands scattered throughout. The secretions

from these glands lubricate the epithelial surface. This is also where the correspondingly named submucosal nerve plexus is located. [19]

The last two layers are the tunica muscularis which consists of inner circular and outer longitudinal smooth muscle layers and the tunica serosa which is loose connective tissue covered by mesothelium. The myenteric plexus is located in the connective tissue between the inner circular and outer longitudinal muscle layers. [19]

**Enteric Nervous System (ENS)**

The Enteric Nervous System (ENS) is an extension of the Peripheral Nervous System (PNS), which innervates the gastrointestinal tract. [21] It regulates endocrine and exocrine secretions, motility, and microcirculation in the gastrointestinal tract.[22] Its primary targets are smooth muscle cells, mucosal secretory cells, gastrointestinal endocrine cells, microvasculature, and immunomodulatory and inflammatory cells.[23]

The neuronal cell bodies of the ENS are assembled into small ganglia. Clusters of nerve processes forming two major plexuses, the myenteric (Auerbach's) plexus and the submucosal (Meissner's) plexus, connect the ganglia. The myenteric plexus is located between the inner circular and outer longitudinal smooth muscle layers of the tunica muscularis which span the entire length of the gastrointestinal tract (Figs. 3 and 4). Its principal functions are to supply these muscle layers with motor innervation and to supply the mucosa with secretomotor innervation.[24] It also sends various projections to the submucosal ganglia, the enteric ganglia of the gallbladder and pancreas, and to the sympathetic ganglia.[23]

Between the inner circular muscle layer and the lamina muscularis is the submucosal plexus which is located within the tela submucosa (submucosal layer). It is most extensive in the small intestine where it has an integral role in secretory control. It is responsible for innervating the glandular epithelium, lamina muscularis, intestinal endocrine cells, and submucosal vasculature where its major roles are regulation of secretion, absorption, and blood flow. [23, 24] The myenteric plexus is far larger than the submucosal plexus with the latter containing less neurons and thinner interganglionic attachments as well as less neurons per ganglion. [25] Myenteric neurons can be labeled with β-III tubulin, which is used as a neuronal marker because it is expressed almost exclusively in neurons.

The structure and function of the ENS differ from other regions of the PNS. The ENS is unique in its ability to mediate reflex behavior without input from the CNS- although CNS influences can often initiate or modify gastrointestinal reflexes. [24, 25] This "law of the intestine", which was proposed by Bayliss and Starling and later confirmed by Trendelenburg essentially says that a "local nervous mechanism" within the gut mediates a reflexive wave of peristalsis in response to luminal distention of the bowel. This reflex was observed even after all extrinsic nerves had been severed. The ENS is able to mediate reflex activity because it contains all of the necessary components: sensory receptors, primary afferent neurons, interneurons, and motor neurons (to propagate peristaltic waves down the gastrointestinal tract). Additionally, the ENS has secretomotor neurons which communicate with the immune system and the CNS. [25]

For many neurons of the ENS, the CNS has no direct role in innervation. Almost none of the efferent vagal fibers synapse directly on ganglia of the submucosal plexus.[25]

This suggests that submucosal ganglion-mediated vagal secretory effects could be indirectly activated via the myenteric plexus. It has been confirmed experimentally that the myenteric plexus is, in fact, the primary target of vagal innervation to the gastrointestinal tract. In contrast to vagal efferents, vagal afferents extend from the smooth muscle, the submucosa, mucosa, and myenteric plexus. Previous work classified the ENS as the "third division of the autonomic nervous system" because it cannot be classified in the same manner as the sympathetic (SANS) and parasympathetic (PANS) divisions of the autonomic nervous system. What distinguishes the SANS and PANS from one another are their respective thoracolumbar and craniosacral origins within the CNS. The ENS, which contains many neurons that lack a direct connection to the CNS, cannot be classified in such a way.[25]

The ENS differs from the PNS in other ways. For example, the ENS lacks endoneurium, the connective tissue layer which ensheaths neuronal cell bodies and axons in most nerves and ganglia of the PNS. It has also been described as having "brain-like" features because its structure is similar to that of the brain. [25] It contains approximately 100 million neurons (about the same number as in the spinal cord). [23]

The vasculature that supplies the myenteric plexus are not fenestrated like those of the rest of the wall of the gastrointestinal tract, but resemble the capillaries of the brain; they are significantly less permeable and form a barrier restricting access of circulating macromolecules to the ENS. [25]

There are many chemical mediators that regulate ENS function. These include over 20 neurotransmitters such as acetylcholine, serotonin, ATP, γ-aminobutyric acid

(GABA) substance P, vasoactive intestinal polypeptide (VIP) and nitric oxide (NO). Most ENS neurons contain several neurotransmitters and many neurons may utilize the same neurotransmitter to carry out different functions. [23]

As mentioned above, there are intrinsic afferent neurons, interneurons, and motor neurons in the ENS.[23] There are two main morphological forms of ENS neurons (as classified by Dogiel): Type I and Type II.[25] Type I neurons are usually flattened in the plane of the ganglion in which they lie. They are also characterized by oval-shaped cell bodies, a single long process which is probably an axon, and many club-shaped dendrites projecting from the cell soma. Type II neurons have more spherical cell bodies which give off multiple long processes so that no one process can be distinguished as an axon. [25]Type II neurons, located in both the myenteric and submucosal plexuxes, are the intrinsic afferent neurons that compose the sensory limb of all intrinsic motor and secretomotor reflexes. They radiate out to interneurons in surrounding plexuses. The interneurons are located between the primary afferent and motor neurons. Those that participate in motor reflexes are termed ascending or descending based on an oral or aboral direction, respectively. Due to the larger after-hyperpolarization that sensory neurons experience which inhibits further excitation, they are called AH neurons.[23] This prolonged hyperpolarization is due to $Ca^{2+}$-activated $K^{+}$ conductance. [25] They receive a slow excitatory synaptic input and are all cholinergic. Enteric motor neurons have type I morphology. The circular muscle has both excitatory and inhibitory motor neurons; the excitatory motor neurons primarily release acetylcholine and substance P and extend locally or orally to the circular muscle while the inhibitory motor neurons, which project caudally into the circular muscle, release VIP and NO. [23]

Although the ENS can function on its own, the CNS does play a significant role in regulating its function. Motor input from and sensory input to the CNS is provided by both the SANS and PANS. The vagal nerves, which innervate the motor and secretomotor functions of the cranial gastrointestinal tract, and the sacral nerves, which innervate the distal colon and rectum, compose the parasympathetic motor pathways. They have extensive connections to the myenteric neurons of the distal colon and rectum (but very little in the small intestine and proximal colon). The extent of direct innervation reflects the amount of control the CNS has over a particular region. All of the preganglionic neurons are cholinergic and excite enteric neurons via nicotinic and muscarinic receptors. [23] The sympathetic fibers entering the GI tract are adrenergic postganglionic fibers whose cells bodies are in the prevertebral ganglia. They synapse on VIP-containing secretomotor neurons, submucosal blood vessels, and gastrointestinal sphincters. [23]

The primary afferent neurons that convey sensory information are carried in the vagal and splanchnic nerves. The cell bodies of vagal primary afferent neurons are located in the nodose ganglia. These neurons can respond to mechanical (gut distention) and chemical (luminal concentrations) stimuli. Splanchic primary afferent neurons have their cell bodies in the dorsal root ganglia and their endings in the gut wall. These neurons sense pain (nociceptors) in the gastrointestinal tract. [23]

Neurons of the ENS, other peripheral sites, CNS can be identified by their expression of neuron-specific beta III tubulin.[26] Tubulin is a member of a small family of globular proteins which polymerize to form microtubules. Functions of microtubules include the growth of processes in developing neurons, centriole structure, and organelle

and secretory vesicle movements.[19] α- and β-tubulin are the most common members of the tubulin family and there are several different isoforms of each. β-III tubulin is one of two isoforms that is specific to neurons and is thus used as a neuronal marker.[26]

**Enteric Glial Cells**

In addition to neurons, the ENS also contains specialized supporting cells called Enteric Glial Cells (EGCs) which outnumber enteric neurons four to 10 times (Fig. 4).[27, 28] EGCs originate from neural crest progenitor cells- most from an early wave of crest cells that yields both neurons and enteric glia.[29] These cells are morphologically different from Schwann cells of the PNS that form the myelin sheath surrounding axons. EGCs are much more irregularly-shaped and closely resemble the astrocytes of the CNS in morphology and ultrastructure. [22-24, 27] Additionally, EGCs don't ensheath individual axons like Schwann cells, but instead surround whole bundles of axons. [25] EGCs provide structural support for neurons and nerve bundles. [22, 23] Like astrocytes of the CNS, EGCs contain 10-nm gliofilaments which are comprised of glial fibrillary acidic protein (GFAP) (Figs. 5 and 6). [25] Therefore, GFAP is used as a marker for both populations of cells (Fig. 6) GFAP is part of the intermediate filament family (type III) and supports glial cell structure.[20, 30] S-100β is the other major marker for EGCs. [28]

EGCs do not manufacture a basal lamina, however a basal lamina does encircle the avascular enteric ganglia and neural attachments.[25] Gap junctions connect them to one another and to enteric neurons much like the coupling of astrocytes in the CNS. [22, 24] EGC processes terminate in swellings that resemble the endfeet of CNS astrocytes and can

project to the vasculature and mucosa. [24, 25] In fact, at the microscopic level the sheath investing the myenteric ganglia looks very much like the pial-glial membrane that surrounds the CNS.[27] Unlike the pial-glial membrane, however, the enteric glial sheath is incomplete, only partially segregating the myenteric neurons and the connective tissue outside the ganglion. [27]

In addition to the morphological similarities between EGCs and CNS astrocytes, there have also been reports about their functional similarities. EGC ablation studies have demonstrated the role EGCs play in neuroprotection and maintaining integrity of the epithelial layer.[22] In two transgenic mouse models, EGC ablation resulted in disruption of the epithelial barrier suggesting that EGCs have a function in maintenance or permeability of the mucosa. The observation that EGCs ensheath neuronal processes involved with regulation of mucosal secretions and extend processes to the mucosal crypts and tips of the villi agrees with this theory. [22] Crohn's Disease patients can exhibit increased intestinal permeability. [21] It has yet to be elucidated, however, whether the role of ECGs in the regulation of epithelial permeability is direct or indirect (via their neuroprotective functions). Also noted in the aforementioned transgenic models, were early aberrations in the vasculature of the intestinal mucosa and submucosa which suggests that EGCs may help regulate gastrointestinal vascular function. [22]

Miampamba *et al.* showed that EGCs can communicate directly with enteric neurons induced Fos expression in neurons and EGCs after stimulation of enteric neurons via vagal cholinergic inputs.[31] As mentioned previously, EGCs have a neuroprotective role. In one of the transgenic murine models, EGC-specific ablation was connected with a marked degeneration of myenteric neurons. [22]

Aside from structural neuroprotection, EGCs also produce neurotrophic factors in the gut- namely, glial-derived neurotrophic factor (GDNF) which supports proliferation and survival of neural-crest derived neurons.[22] It has also been postulated that EGCs may act as an intermediary between enteric neurons and the general circulation, helping to regulate the exchange of metabolites and nutrients. EGCs are glycogen-rich which may mean that they provide a major source of glucose for enteric neurons such as astrocytes do for CNS neurons.[22] They are also the only cells in the ENS that are immunoreactive for L-arginine, which is necessary for the synthesis of NO.[22] NO is a transient free radical gas produced by endothelial cells that relaxes vascular smooth muscle to cause vasodilation.[32] Thus enteric neurons may get their L-arginine from EGCs as needed. L-arginine is mainly stored within astrocytes in the CNS. It has also been theorized that EGCs regulate perineuronal ion concentrations, buffering extracelluar $K^+$ ions. When $K^+$ ions are released during neuronal activity, EGCs take it up through potassium channels and reallocate it into areas of low extracellular $K^+$. CNS astrocytes work in a similar fashion to prevent possible excitotoxicity of neurons from increased $K^+$ in extracelluar space surrounding the synapse.[22] EGCs are also immunoreactive for SP and neurokinin A (also called substance K).[33] SP is a member of the tachykinin family of neuropeptides produced by sensory nerve fibers.[32] Its functions include modulation of smooth muscle contraction, neuro-neuronal transmission, vasodilation, and enteric secretion. It also plays a role in neurogenic inflammation and nociception.[34] Neurokinin A is another tachykinin and a strong contractile agonist for colon circular muscle in humans. [35]

In addition to their supportive and neurotrophic roles, astrocytes in the CNS have other roles such as secreting cytokines, scavenging neurotransmitters and toxic

substances, and even acting as antigen-presenting cells. [24] EGCs apparently also exhibit some of these same immunological functions. EGCs may have a possible role in modulating inflammatory responses because they produce interleukins and express major histocompatibility complex class II antigens in response to simulation by cytokines.[24] Major histocompatibility complex II plays an important role in antigen presentation and in the communication between antigen-presenting cells and T-lymphocytes. [34] A proliferation of EGCs (gliosis) has been observed in patients with ENS inflammatory disease. [22]

**Expression and Potential Role of MC4R in the Gastrointestinal Tract**

The expression of MC4R mRNA and protein has been shown in some regions of the gastrointestinal tract. A study using MC4R-green fluorescent protein (GFP) reporter mice indicated the presence of MC4R in the stomach myenteric ganglia and in the myenteric ganglia, submucosal ganglia, and mucosa of the duodenum.[1] The hepatic vagus nerve and left cervical vagus nerve also had evidence of MC4R-positive fibers, indicated by immunoreactivity for the green fluorescent protein (GFP) reporter gene. Neurons expressing cholecystokinin A receptor (CCKAR) co-expressed MC4R although many MC4R-expressing neurons did not express cholecystokinin A receptor. This work suggests that the MC4R-expressing vagal afferents modulate satiety signals, such as cholecystokinin, originating from the gut, where as MC4R-expressing vagal efferents which innervate the liver and myenteric plexus may help regulate certain functions including glucose homeostasis, motility, and secretion. [1] MC4R may also play a role in

attenuating inflammation in the gastrointestinal tract as associated with inflammatory bowel disease (IBD). Treatment with α-MSH has been shown to reduce inflammation in experimentally-induced IBD and there is a possible role for MC4R in this mechanism.[36, 37]

**Inflammation**

Inflammation is an innate response to injury. It can be categorized as acute or chronic. Acute inflammation is associated with leakage of fluid and plasma proteins from circulation into the tissues (exudation), whereas chronic inflammation involves leukocyte infiltration of the injured tissue as well as proliferation of fibroblasts and small blood vessels. There is no clear distinction between acute and chronic inflammation, but unresolved acute inflammation will eventually lead to chronic inflammation, which is usually characterized as a state of inflammation that lasts more than a few days or weeks.[32]

Several factors are typical of a chronic inflammatory response: proliferation of fibroblasts and vascular components and infiltration of lymphocytes, macrophages, and plasma cells. Causes of chronic inflammation may include infection, remains of dead organisms, foreign bodies, and products of metabolism. Most chronic inflammatory reactions are coordinated by the CD4+ T cell. [32] Many diseases are associated with chronic inflammation such as obesity, arthritis, Alzheimer's disease, Parkinson's disease, and Inflammatory Bowel Disease (IBD).[38-42]

**Inflammatory Bowel Disease in the Colon**

Inflammatory Bowel Disease (IBD) is a chronic condition that plagues approximately 1.4 million Americans. The peak onset is in individuals between 15 and 30 years old. [43] Ulcerative Colitis and Crohn's Disease are the two major types of IBD. Ulcerative colitis affects the rectum and may affect the colon. It can be classified according to the extent of colonic involvement. Proctitis refers to colitis restricted to the rectum where as proctosigmoiditis involves the area from the rectum to the sigmoid flexure (portion of the left colon located in the pelvis that extends from the distal colon to the rectum); these are referred to as "distal" disease. "Left-sided" colitis involves the area up to the splenic flexure while "extensive" colitis involves the area up to the hepatic flexure. Pancolitis is the most extensive form affecting the entire colon.[44] In most cases, it extends proximally in a convergent manner. Tissue damage in the wall of the gut is usually limited to the mucosa and lamina propria.[43]

Crohn's disease can affect any part of the gastrointestinal tract although it usually targets the ileum and/or colon. It exhibits patches of affected and non-affected areas characterized as skip lesions. Tissue damage may extend the entire thickness of the gut wall including the tunica muscularis. [43, 45] Both ulcerative colitis and Crohn's disease are generally chronic and recurring. Ulcerative colitis can be cured by surgical removal of the colon but there is no known cure for Crohn's disease. [46]

Symptoms of IBD include: abdominal pain, bloody diarrhea, urgency, loss of appetite, weight loss, fever, and anemia. [44, 46] Another notable complication is the increased risk of colon cancer; a 1994 study concluded that there were 18-fold and 19-

fold increased risks above that of the general public in developing colorectal cancer in patients with extensive Crohn's disease and ulcerative colitis, respectively.[47] The risk of colorectal cancer was increased in both types of IBD for individuals whose colitis began before 25 years of age.[47]

In addition to genetic predisposition, researchers have proposed that IBD results from the presence of normal luminal flora or other environmental factors stimulating the improper and continuous activation of the mucosal immune system. The probable cause of this abnormal response is a combination of defective immune system and intestinal epithelial barrier function.[48] Other factors have been implicated in the development of IBD. These include the use of nonsteroidal anti-inflammatory drugs, which can induce disease flares, as well as changes in diet and antibiotic use. [43, 48] What has yet to be elucidated is what activates the immune system: an inherent defect such as constitutive activation, the malfunction of a down-regulatory mechanism, or sustained stimulation after an alteration in the epithelial mucosal barrier. [48]

In IBD, activated macrophages produce several inflammatory cytokines, including TNF, interleukin-1 (IL-1), and interleukin-6 (IL-6). [43, 48] Inflammation is then maintained by recruitment of additional leukocytes from the vascular space to diseased areas, which depends on the expression of adhesion molecules in the local microvasculature.[32]

In their review, Bouma and Stroma identify "two pathways to mucosal inflammation" that are almost always responsible.[49] These are: either an extreme Th1-cell response, associated with elevated secretion of IL-12, interferon-γ (IFN-γ) and/or TNF-α,

or an extreme Th2-cell response. Th2-cells synthesize and secrete IL-4, IL-5, and/or IL-13. This delineation is evident in the cytokine profile observed following induction of intestinal inflammation by the agents TNBS and oxazalone. TNBS produces an IL-12 mediated response characterized by cellular infiltrate that extends through the entire thickness of the wall and is sometimes associated with granulomas .[49] This response can be abated by anti-IL-12 antibodies as IL-12 promotes the generation of Th1 cells. [32, 49] Oxazalone, however, causes inflammation characterized by a more superficial cellular infiltrate and more damage to the epithelial layer. Natural-killer T cells are induced to produce IL-13. The development of colitis is prevented when natural killer T cells are eliminated or IL-13 is blocked. While the histopathological profile of Crohn's disease resembles that of experimental Th1-cell-mediated colititis, the histopathological profile of ulcerative colitis is more similar to experimental Th2-cell-mediated colitis. [49]

**Role of Luminal Flora in IBD**

As mentioned above, luminal flora have been identified as playing a role in the pathogenesis of IBD.[48] The microbiota that live within the lumen of the gastrointestinal tract are involved in the development of the intestinal immune system, provide important nutrients, and modulate energy metabolism. An individual obtains gut flora at birth. During the first year of life they change quickly but by adulthood each person has a unique and rather stable population of fecal flora. Changes in this population occur in disease states and in response to environmental and developmental factors. [43] The presence of luminal flora is generally agreed upon to be a compulsory and integral factor

in the development of IBD. This belief has been backed by various studies in murine models of colitis created by genetic manipulation. The presence of luminal flora is an apparent prerequisite for the development of "spontaneous" colitis in rats and mice. In fact, when several mutant strains were maintained in a germ-free environment, colitis did not occur, but it did quickly develop following colonization by bacteria. [48] Antibiotics are an affective treatment for some IBD patients.[43] Furthermore, studies in IBD patients showed an increased number of surface-adherent and intracellular bacteria in their colonic epithelium. [48]

One theory is that in genetically predisposed individuals, luminal flora act as antigens or co-stimulatory factors which fuel an inflammatory response. However, there is a class of beneficial microflora called "probiotics" that improve inflammation possibly by inducing suppressor cytokines. [49]

**Role of Intestinal Epithelium in IBD**

The integrity of the epithelial lining of the gastrointestinal tract is another important factor in the development of IBD. The gastrointestinal lumen is directly exposed to many different antigens coming from the external environment through the ingestion of food. Therefore, it has a vital role in the mucosal immune response. The intestinal epithelial cells physically prevent excessive passage of bacteria and other antigens to the circulation from the gastrointestinal lumen. Intercellular junctions and tight junctions are responsible for keeping the mucosal barrier intact but in IBD these

components are compromised. Such abnormalities may be from a primary deficiency in barrier function or a secondary result of inflammation. [43]

Specialized epithelial cells such as goblet cells and Paneth cells provide further defense against bacterial invasion. Goblet cells secrete mucus which covers the epithelium and reduces contact between bacteria and epithelial cells.[50] Paneth cells (absent in the colon) also provide defense against bacterial invasion by secreting antimicrobial peptides like α-defensin and lysozyme but there are no Paneth cells in the colon.[19, 43, 50] Stem cell renewal of epithelial cells and repair of the epithelial lining are processes that manage and eventually stop intestinal inflammatory response to insult. [43, 51] However, in IBD, inflammation usually leads to continuous epithelial insult resulting in erosions, ulcerations, and decreased defensin secretion. This allows for more exposure to intestinal flora, only intensifying the inflammatory response. Several types of epithelial dysfunction such as abnormal epithelial-cell development, proliferation, barrier function, or epithelial restitution after injury can cause intestinal inflammation in mouse models of IBD. [43]

**Inflammation and Enteric Neuropathy**

The ENS has the ability to modify itself adaptively in an effort to alleviate symptoms and improve disrupted gastrointestinal function. Abnormalities that IBD patients often face, such as altered secretion and sensory-motor function, are indicative of the effect that inflammation has on the ENS. [34]

A number of studies have linked IBD with changes in ENS morphology and corroborating evidence suggests a potential role for the ENS in the pathogenesis of IBD. IBD may be aggravated by psychological stress, which might indicate important interactions between the brain, ENS, and gastrointestinal tract. A study involving TNBS-induced colitis in rats showed a reduction in severity of the colitis followed by topical and subcutaneous administration of lidocaine suggesting that lidocaine may block enteric neural pathways of gastrointestinal inflammation by hindering neural transmission. [23]

Hypertrophy or hyperplasia of ganglia and nerve bundles as well as EGC hyperplasia have been documented in tissue analysis of ulcerative colitis and Crohn's disease patients.[34] In Crohn's disease patients, nerve trunk hyperplasia and hypertrophy was chiefly observed in the mucosa, submucosa, and myenteric plexus of the ileum and colon. These findings were directly proportional to the degree of inflammatory infiltrate. Such findings are not as common in the mucosa and submucosa of ulcerative colitis patients. A characteristic of both Crohn's disease and ulcerative colitis is the existence of plasma cells, lymphocytes, and mast cells within the submucosal and myenteric plexuses and near nerve fibers. [34]

Observations of the ultrastructure of tissue samples from patients with ulcerative colitis and Crohn's disease indicated swollen and empty axons that were filled with large membrane-bound vacuoles, swollen mitochondria, and concentrated neurofibrils. Among Crohn's disease samples, these irregularities were observed in affected and non-affected areas. [34] A study by Sanovic *et al.* involved dinitrobenzene sulfonic acid (DNBS) – induced colitis in male rats. The authors found that within 24 hours, inflammation had caused the significant loss of ganglia (but not neurons) from the submucosal plexus and

loss of neurons (but not ganglia) from the myenteric plexus. [52] EGCs also exhibit changes in IBD. Tissue specimens from Crohn's disease patients showed that EGCs had increased surface-expression of major histocompatibility complex class II antigens. [34]

There are also modifications in the release of neurotransmitters and the receptors for these neurotransmitters in IBD. One of the main neurotransmitters contained in enteric neurons is substance P. An observation of tissue samples from ulcerative colitis patients showed over three times as many, in affected areas, and over twice as many, in non-affected areas, substance P-positive myenteric neurons compared to controls. Many of these substance P-positive myenteric neurons are also colocalized with choline acetyltransferase-positive cholinergic neurons.[53] This could account somewhat for the disturbed gastrointestinal motility that ulcerative colitis patients experience because substance P activates colonic motility in rats. [53, 54] In a rat model of TNBS-induced colitis, substance P-receptor antagonists decreased the infiltration of granulocytes. [23] There is contradictory evidence as to whether an increase in substance P-immunoreactivity is also increased in Crohn's disease. [23, 53]

Various types of enteric neurons, such as inhibitory motor neurons, descending interneurons, vasomotorneurons, and secretomotorneurons, contain VIP. It regulates smooth muscle relaxation and neurally-mediated secretion by enterocytes. [34] It also decreases intestinal paracellular permeability, increases peristaltic action of small and large intestines, and stimulates the gastrointestinal tract to eliminate water and ions. [20, 28] In the ulcerative colitis study mentioned in the previous paragraph, the proportions of choline acetyltransferase and VIP containing neurons were not significantly different among inflamed and non-inflamed segments and controls. [53] In contrast, two studies

observed an increase in VIP-positive submucosal and myenteric neurons in the inflamed and non-inflamed tissues of Crohn's disease patients. [34]

In line with the idea that structure determines function, the morphological and chemical changes that take place within the ENS frequently accompanied by degeneration of ganglion cell and nerve processes and necrosis, inevitably lead to a disturbance in function. One group studied the effects of Trinitrobenzene sulfonic acid (TNBS) - and *Trichinella spiralis*-induced colitis on myenteric nerve function in inflamed and noninflamed regions in the rat. The study assessed functional changes by measuring myeloperoxidase activity (MPO) from whole tissue and $^{3}H$ from longitudinal muscle-myenteric plexus (LMMP) preparations.[55]MPO is a neutrophil-specific peroxidase enzyme predominantly present in neutrophils that can be used to assess the amount of neutrophil infiltration in tissues because MPO content is directly proportional to neutrophil number.[56] $^{3}H$ release, measured after KCl or electrical field stimulation, reflects the amount of norepinephrine released from adrenergic nerves within the myenteric plexus. They found that both the TNBS and *T. spiralis* model had significant increases in MPO as well as marked reduction of $^{3}H$ release. Interestingly, they also found that suppression of $^{3}H$ release from myenteric nerves occurred in non-inflamed areas of the large and small intestine as well as the inflamed distal colon. This suggests a systemic factor of the inflammatory process. Supportive findings have been observed in the bowel of Crohn's disease and ulcerative colitis patients. [55]

**Current Therapies for IBD**

IBD is diagnosed based on a combination of history, presentation, endoscopic, radiologic, and histologic findings, and routine laboratory tests. In their blood serum, 70 percent of ulcerative colitis patients have perinuclear-staining anti-neutrophil cytoplasmic antibodies present and 50 percent or more of Crohn's disease patients have anti-*Saccharomyces cerevisiae* antibodies. Crohn's disease is indistinguishable from ulcerative colitis in as many as 10 percent of patients with IBD that is restricted to the colon. [48]

There is no known cure for IBD, but several therapies are presently used to manage the disease. Today's treatments for IBD aim to inhibit proinflammatory cytokines, the migration of cells into intestinal tissues, and T-cell activation and proliferation.[43] Current treatments for IBD include: 5-Aminosalicylic acid, corticosteroids, immunosuppressive and immunoregulatory agents, anti-TNF therapy, antibiotics and prebiotics, as well as other experimental agents. [48]

The proposed mechanism of action of 5-Aminosalicylate is inhibition of the production of prostaglandins and leukotrienes, preventing bacterial peptide-induced chemotaxis, scavenging reactive oxygen species, and possibly inhibiting the action of NF-κB by acting on the epithelial cells. Benefits include the reduction of colorectal cancer risk by as much as 75% but common side-effects include headache, nausea, epigastric pain, and diarrhea.[48]

In cases where 5-aminosalicylate-based compounds are insufficient, corticosteroids such as hydrocortisone and prednisone are used to treat IBD. [48]

Corticosteroids work by inhibiting various inflammatory pathways. They suppress transcription of interleukins and arachidonic acid metabolism, induce IκB, the inhibitory subunit of nuclear factor κB (NF-κB) to inhibit NF-κB, and stimulate apoptosis of lymphocytes in the lamina propria of the GI tract. [44] Some early side effects include acne, edema, sleep and mood disturbance, and diabetes. Side effects include osteoporosis, myopathy, and vulnerability to infection after prolonged treatment (more than 12 weeks). Effects of withdrawal include acute adrenal insufficiency and arthralgia (joint pain).[44, 48]

Immunomodulatory drugs are commonly used to treat ulcerative colitis and Crohn's disease patients in whom the corticosteroid dose cannot be lowered or terminated, allowing gradual reduction in corticosteroids and prolonged remission. Azathioprine and mercaptopurine are the most widely used drugs of this class and their mechanisms of action have yet to be elucidated but possibly involve inhibiting the generation of a specific and enduring group of T cells. Patients given these drugs are at an increased risk for opportunistic infections.[48]

Infliximab (IFX) is an anti-TNF drug that has been very effective in the treatment of Crohn's disease patients. Its mechanism of action is not entirely known but it could depend on apoptosis of inflammatory cells. Infliximab is a chimeric monoclonal antibody that binds to soluble TNF. [44, 48]

Clinical evidence has shown antibiotics to be beneficial in the treatment of some Crohn's disease patients but not in ulcerative colitis patients. This implies a differential role of luminal flora in these two conditions. Metronidazole can repress colonic Crohn's

disease, but one significant side effect is neurotoxicity. The use of probiotics ("healthy" bacteria) is another promising therapy that would circumvent systemic side effects. [48]

Other experimental agents have been and continue to be investigated for the treatment of inflammatory bowel disease including antibodies against specific cytokines, growth hormone, transdermal nicotine, fish oil, and rosiglitazone. [48]

**α-MSH and Inflammation**

The neuropeptide α-MSH has an important function in the regulation of immune and inflammatory reactions and, because it has no known pharmacologic toxicity, it has potential for the treatment of various inflammatory diseases, such as IBD. [3] α-MSH has anti-inflammatory effects on acute, chronic, systemic, allergic, and CNS inflammation. [37] This is true for both α-MSH and its C-terminus fragments (which include N-acetlyated and C-amidated tripeptide KPV and various stereoisomers). [12] α-MSH is found in the pituitary, brain, and some peripheral tissues. It can exercise its anti-inflammatory effects through centrally expressed MCRs, or on immune cells and resident non-immune cells of peripheral tissues.[3]

Multiple research efforts have concluded that α-MSH inhibits the production and action of proinflammatory cytokines, such as IL-1, IL-6, IL-8, TNF-α, IL-2, IFN-γ, and monocyte chemoattractant protein-1 while stimulating the secretion of anti-inflammatory cytokines such as IL-10. Additionally, α-MSH inhibits inducible nitric oxide synthase (iNOS) to reduce NO production by macrophages. It significantly reduces the number of inflammatory cells migrating to areas of injury. Furthermore, α-MSH has been

demonstrated in vitro to down-regulate pro-inflammatory cytokine production in response to endotoxin by direct action on MCRs on peripheral immune cells as well as to inhibit peripheral inflammation (in the skin) following icv injection. [3, 6, 12, 57]

Studies demonstrating α-MSH's capacity to suppress expression of TNF-α have involved various cell types including human astrocytes, microglia, monocytes, and macrophages. [12] The observation that there is an upregulation of the POMC gene and increased production of α-MSH upon stimulation of monocytes with enodotoxin or cytokines suggest that α-MSH exerts anti-inflammatory effects on monocytes/macrophages in an autocrine fashion. It has been proposed that the enhancement of cAMP is responsible for most of α-MSH's inhibitory effects. [57] For example, the enhancement of cAMP suppresses the activation of inflammatory factors such as NF-κB. This pathway is a probable mechanism for some of the anti-inflammatory effects of α-MSH. Nanomolar doses of α-MSH suppress NF-κB activation in various cell types in response to TNF-α, LPS, and IL-1. Deactivation of NF-κB by α-MSH is associated with prevention of the degradation of IκBα. [12]

Furthermore, α-MSH has been shown to attenuate inflammation and prevent lipopolysaccharide (LPS)- and IFN-γ-induced apoptosis in rat astrocytes via MC4R. Astrocytes are the most abundant glial cell type in the brain and have many important roles in the support of neurons. They provide metabolic and trophic support for neurons by supplying growth factors and neurosteroids and have a large role in regulation of the extracellular ionic environment. They also have a neuroprotective role, protecting neurons from oxidative stress and excitotoxicity. When astrocytes are activated in situations such as disease, infection, and ischemia, an inflammatory response ensues

causing the production of inflammatory mediators. Such inflammatory mediators as cytokines, lipopolysaccharide, and NO can induce apoptosis in astrocytes leading to neurodegeneration and neuropathology. As mentioned, α-MSH inhibits the release and/or action of these cytokines. Rat astrocytes express MC4R and, similar to the lipopolysaccharide-induced fever study, MC4R blockade with the selective antagonist HS024 inhibits the anti-inflammatory affects of α-MSH. This further supports the role of MC4R in the prevention of induced astrocyte apoptosis. [58]

The ability of α-MSH to quell fever via central administration is well known. Serum levels of α-MSH have been shown to increase during endotoxemia, after administration of pyrogens, and in inflammatory conditions.[3] There are several reasons to infer that the antipyretic effects of α-MSH are mediated by MC4R. First, MC4R mRNA-expressing cells have been localized in preoptic, hypothalamic, and brainstem nuclei; these areas are involved in thermoregulation and fever. [3] Second, suppression of lipopolysaccharide-induced fever in rats by icv α-MSH was blocked by an equimolar dose of HS014, an MC4R antagonist, which was co-injected with the agonist.[59]

In addition to fever, α-MSH has been shown to attenuate inflammation in other experimentally-induced inflammatory models such as arthritis, airway inflammation, and pancreatitis. Central administration of α-MSH has been shown to attenuate peripheral inflammation. One study supporting this concept involved observing the effect of central α-MSH or saline on inflammation by measuring inflammatory markers in the circulation, lungs, and livers of mice that had been injected intraperitoneally with lipopolysaccharide. Indeed, initial increases in circulating levels of TNF-α and NO by lipopolysaccharide were reduced by central administration of a minute concentration of α-MSH. Levels of

iNOS were also reduced in the lungs and liver. When central α-MSH was blocked, there were considerably higher concentrations of circulating pro-inflammatory markers induced by lipopolysaccharide. [57]

One important implication of α-MSH's anti-inflammatory properties is its potential use in the treatment of IBD. TNF-α and NO, which have both been associated with IBD, are thought to be directly responsible for or indicative of local inflammatory responses. α-MSH has been shown to decrease the levels of these inflammatory markers. Although there are some reports disputing the involvement of TNF-α in IBD, the majority of evidence supports it. In one study, in situ hybridization for TNFα mRNA was greater in lamina propria cells of mucosal samples from IBD patients compared to controls. In another study, eight out of ten Crohn's Disease patients treated with a single dose of anti-TNF human/mouse chimeric monoclonal antibody improved within four weeks. NO is also a key inflammatory marker in IBD. Studies have shown increased iNOS activity in rats with sulfydryl blocker-induced colitis and a 100-fold increase in luminal NO gas of ulcerative colitis patients versus controls. [37]

α-MSH has also been shown to have a potent anti-inflammatory effect in experimentally-induced colitis. In one murine model, colitis was induced by ingestion of 5% dextran sulfate (DSS). Among DSS-treated mice, the appearance of blood in the stool and colitis-associated weight loss was inhibited in those treated with two daily IP injections of 50μg α-MSH. In fact, the gross appearance of the colons from these animals was comparable to those of normal animals. This study also found that TNF-α produced by lower colonic tissue of saline-treated animals was six times greater than that of α-

MSH treated mice and that nitrite produced in the lower bowel of saline-treated versus control was also significantly higher. [37]

A rat model was published in which colitis was induced by intrarectal administration TNBS. In this study, colitis-induced rats were divided into acute (three days) and chronic (seven days) groups. Colonic damage was assessed using a "macroscopic score" based on certain criteria, with 0 meaning there was no damage and 6-10 meaning that the extent of damage was greater than 2 cm along the length of the colon. Microscopic scoring by histological analysis was also done. In both the acute and chronic groups, the macroscopic score of the colitis group was reduced by treatment with α-MSH, but the microscopic score was not. [36]

In summary, α-MSH or α-MSH analogs have the potential for treatment of inflammation in the colon. As a first step toward elucidating the mechanism by which α-MSH attenuates inflammation in the colon, this study sought to identify MC4R expression in the myenteric neurons and EGCs of the colon.

## Table 1. Melanocortin receptors, ligands, locations, and functions

| Receptor | Location | Function | Endogenous Ligand(s) |
|---|---|---|---|
| **MC1R** | Melanocytes, immune cells, glioma cells, and astrocytes | Skin pigmentation and inflammation | α-, β-, and γ-MSH, ACTH, and Agouti* |
| **MC2R** | Adrenal cortex | Adrenocortical steroidogenesis | ACTH |
| **MC3R** | CNS, kidneys, stomach, duodenum, and pancreas | Energy homeostasis, feeding, natriuresis, and inflammation | α-, β-, and γ-MSH, ACTH, and AgRP* |
| **MC4R** | CNS (mainly PVN, DMV), astrocytes, myenteric neurons of stomach and duodenum, myenteric neurons of colon*, enteric glial cells*, and pancreas | Energy homeostasis, male erectile function, female sexual desire, inflammation, and neuroprotection | α-, β-, and γ-MSH, ACTH, Agouti*, and AgRP* |
| **MC5R** | Exocrine cells of Harderian, lacrimal, preputial, and prostate glands | Synthesis and secretion of exocrine products | α-MSH |

***antagonist**

* **Documented by this study**

**Table 2. Primary Antibodies used for Immunohistochemistry**

| Tissue Antigen | Host Species | Dilution | Source |
|---|---|---|---|
| MC4R | Rabbit | 1:200<br>1:750* | Caymen Chemical (Ann Arbor, MI) |
| Neuronal Class III β-tubulin | Mouse | 1:10,000 | Covance Inc. (Princeton, NJ) |
| GFAP | Mouse | 1:200 | DAKO (Carpinteria, CA) |

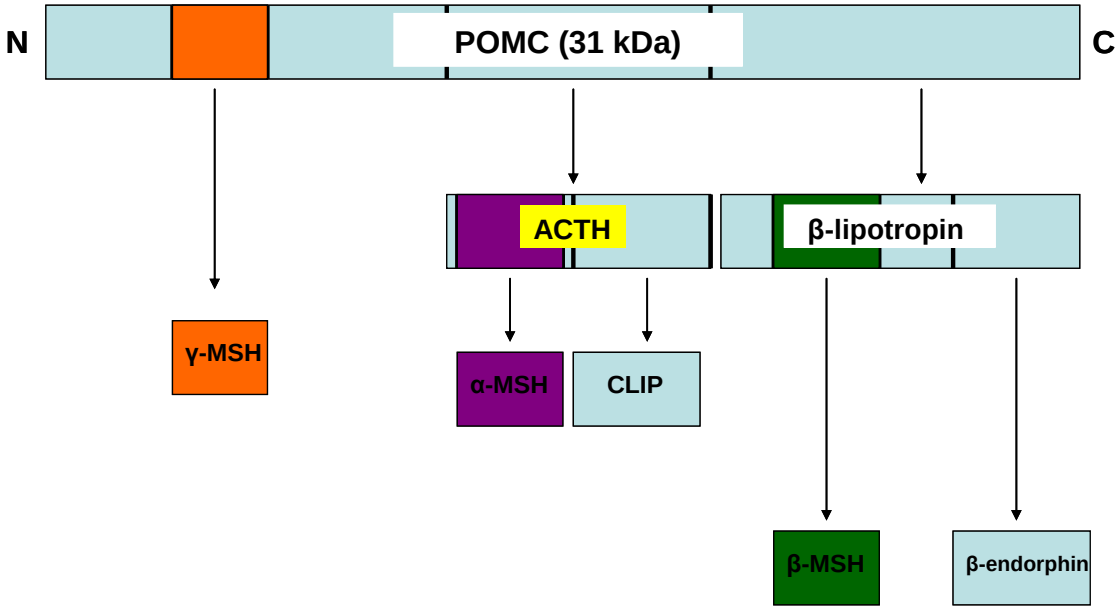
*MC4R/GFAP co-localization

**Table 3. Secondary Antibodies used for Immunohistochemistry**

| Antibody | Dilution | Source |
|---|---|---|
| Goat anti-Rabbit Alexa 488 | 1:500<br>1:800* | Invitrogen (Carlsbad, CA) |
| Goat anti-Mouse Alexa 488 | 1:500 | Invitrogen (Carlsbad, CA) |
| Donkey anti-Mouse Alexa 594 | 1:500 | Invitrogen (Carlsbad, CA) |
| Donkey anti-Rabbit Alexa 594 | 1:500 | Invitrogen (Carlsbad, CA) |

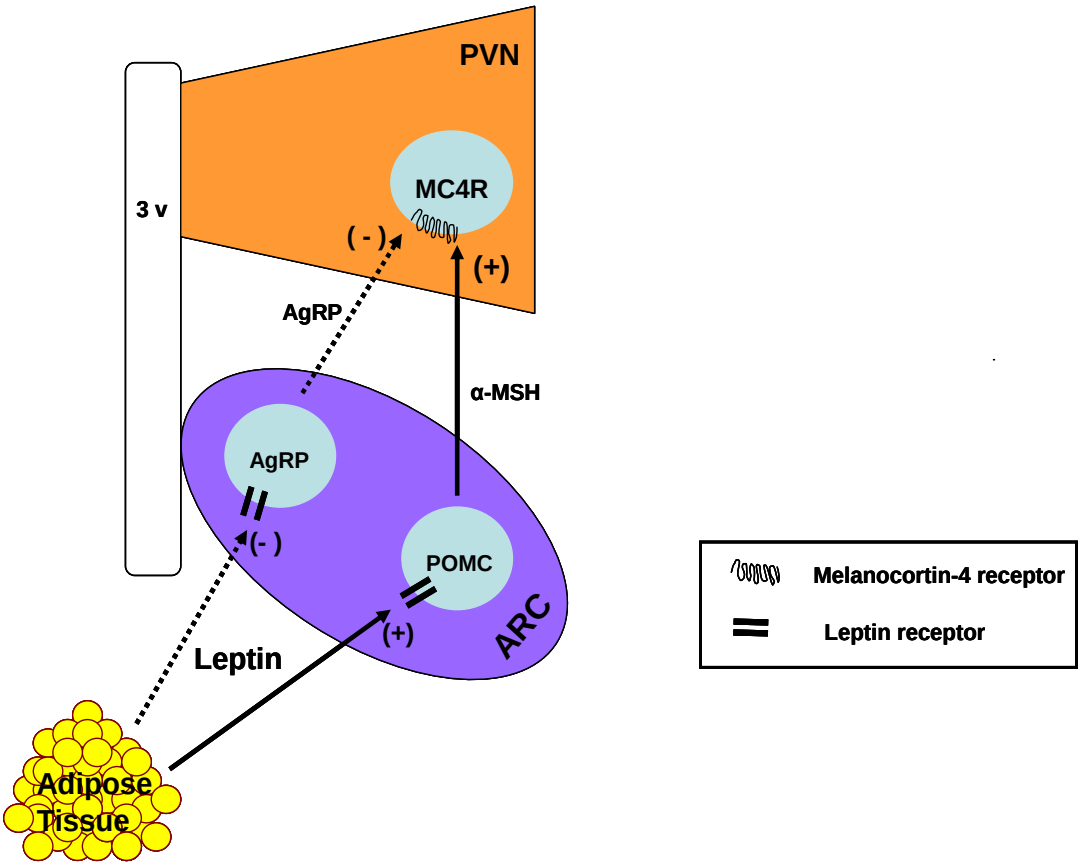
*LMMP sections

## Fig. 1. Post-translational Modification of POMC Yields Melanocortin Peptides



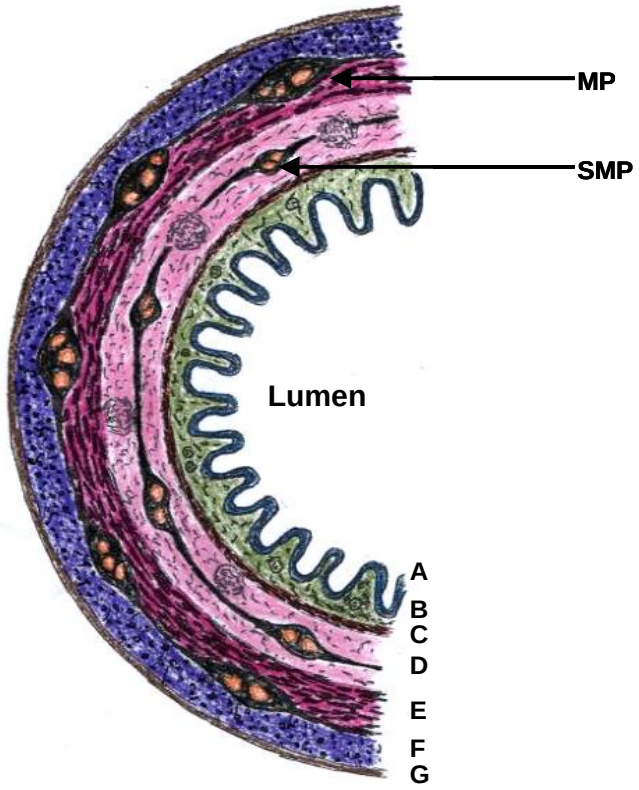

Schematic drawing showing the melanocortin bioactive peptides derived from the prohomone pro-opiomelanocortin (POMC), which is generated in the neurons of the hypothalamus and the corticotroph cells of the anterior pituitary, among other tissues. POMC-derived melanocortin peptides (adrenocorticotropin (ACTH) and α- and β-, and γ-MSH) act as endogenous ligands for the melanocortin-4 receptor (MC4R) which is involved in multiple functions including appetite control, energy homeostasis, inflammation, pain, immune responses, and sexual activity.[60]

**Fig. 2. Leptin Modulates the Melanocortin System**




Leptin released from adipose tissue stimulates POMC-expressing neurons in the arcuate nucleus to release α-MSH and inhibits the release of AgRP. The paraventricular nucleus (PVN) is a major site of MC4R expression in the CNS.[61]

## Fig. 3. Layers of the Wall of the Colon




Cross-section of the colon illustrating the layers of the colon wall. The **tunica mucosa** consists of **(A)** epithelium, **(B)** lamina propria, and **(C)** lamina muscularis. **(D)** denotes the **tela submucosa** (**submucosal layer**), where the submucosal plexus **(SMP)** is located. The **tunica muscularis** consists of the **(E)** inner circular and **(F)** outer lonigitudinal muscle layers, in between which is the myenteric plexus **(MP)**. **(G)** denotes the **tunica serosa** (**serosal layer**).

## Objectives

1) **Delineate expression of MC4R in the colon using semi-quantitative RT- PCR.**

2) **Characterize specific cellular distribution of MC4R in colon using IHC.**

3) **Assess the function of MC4R in these locations by central and peripheral administration of MTII, an MC4R agonist.**

We have learned several important things from the review of the literature. We know that α-MSH has anti-inflammatory properties in the brain as well as various peripheral sites including the colon. While MC4R has been identified as the receptor through which α-MSH attenuates inflammation in the brain, the mechanism by which it quells inflammation in the periphery has yet to be elucidated.[3] We have also learned from various studies that intestinal inflammation is coincident with intestinal neuropathy, which includes changes in enteric neurons and EGCs.[22, 34, 52]

MC4R expression was previously shown in the gastric and duodenal myenteric plexuses but there have not been any studies on the expression of MC4R in the ENS of the lower bowel.[1] If we could identify the presence of MC4R in the colon, and localize it to the ENS, we might shed new light on a possible pathway by which α-MSH exerts anti-inflammatory effects in the colon. We wanted to observe possible expression of MC4R by colonic myenteric neurons and EGCs. The following factors

support the relevance of EGCs in the discussion on MC4R and inflammation: there are morphological and functional similarities between EGCs and astrocytes in the CNS;[27] α-MSH acts on MC4R in astroglia to decrease the expression of inflammatory markers induced by lipopolysaccharide/IFN-γ;[3]ablation of EGCs in the small intestine has been shown to quickly lead to fulminant enteritis;[62] and a proliferation of EGCs (gliosis) has been observed in patients with ENS inflammatory disease much like astrocyte hyperplasia and proliferation (astrogliosis) that occurs in the CNS in response to inflammation.[22]

## Materials and Methods

### Animals

Thirty-eight adult male Sprague-Dawley rats (Harlan Laboratories, Prattville, AL) with weights ranging from 200-300g were used. The original number was 40 but 2 rats died before the experiment from surgical complications. All procedures conformed to NIH guidelines and were approved by the Auburn University IUCAC committee. The rats were divided into five groups according to the type and route of treatment: group 1 received an intracerebroventricular (icv) dose of MTII; group 2 received an icv dose of leptin; group 3 received an intraperitoneal (ip) injection of MTII; group 4 received an icv dose of vehicle; and group 5 received an ip injection of vehicle. Artificial cerebrospinal fluid (artificial CSF) (Harvard Apparatus, Holliston, MA) was used as the vehicle.

### Surgery

Rats in groups 1, 2, and 4 received surgically implanted icv cannulae. Cannulation surgical procedures were performed under aseptic conditions. The rats were anesthetized with an ip injection of ketamine/xylazine and dorsal surface of their heads were shaved. Ketamine was administered at 100mg/kg body weight and xylazine was administered at 1mg/kg body weight. A pedal reflex test was used to ensure that a surgical plane of

anesthesia had been reached and the rats were placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA) with ear bars and a nose piece set at + 5.0 mm. The incision site was prepped with povidone-iodine solution and an incision of approximately two centimeters was made on the dorsal surface of the head down the midline. Lidocaine was applied to the site as a local anesthestic and the connective tissue on the surface of the skull was rubbed off to prevent scarring that could lift the cannula out of place.

The following coordinates, determined by previous experiments, were used to insure the proper placement of the cannula: 1.8 mm posterior to bregma, 0.0 mm lateral to the midline, and 8.6 mm ventral to the skull. A hole was drilled into the skull and a 10 mm long, 22-gauge stainless steel guide cannula (Plastic One, Roanoke, VA) was implanted into the third ventricle. The cannula was secured to the skull with four stainless steel screws and acrylic dental cement. An 11 mm long "dummy" cannula was then placed inside the guide cannula. The top of the dummy cannula was screwed onto the top of the guide cannula to keep it in place. Rats were placed in hanging wire cages and given at least four days to recover.

Angiotensin II Test

Proper cannula placement was confirmed by an angiotensin II test. Rats received an icv 50 ng dose of angiotensin II (American Peptide, Sunnyvale, CA) , dissolved in 6µL of artificial CSF, to stimulate water consumption. Rats that consumed 5mL or more within 15-20 minutes were considered positive for the test, meaning that their cannulas were properly placed. Tests were repeated for rats that were negative for the initial test,

and those that were negative a second time were used in the untreated control group (group 4) or in the MTII-treated ip group (group 3).

Dosing

An automated dosing device consisting of an infusion pump connected to a 1ml syringe, tubing, and a dosing cannula was used for icv infusion of angiotensin II, MTII, leptin, and vehicle. Rats in group 1 were injected via icv with a 10μL of artificial CSF containing 50 ng of MTII. Rats in group 2 were injected via icv with 5 μL of artificial CSF containing 5μg of leptin. Group 4 rats served as the icv control group, and were injected with10 μL of artificial CSF. The total volume of the icv injections was delivered to each rat over a 60 second period.

Rats in Groups 3 and 5 were dosed via ip injections. Group 3 rats were injected with a 100 μL artificial CSF containing 50 ng of MTII. Group 5 rats were injected with 100 μL of artificial CSF.

Perfusion

Rats from which the myenteric plexus were to be isolated were initially anesthetized by isoflurane vapor in a closed chamber. They were then given a 0.4 mL dose of pentobarbital and a 0.6 mL dose of heparin via ip injection. A pedal reflex test was used to ensure that a surgical plane of anesthesia had been reached.

The rat was placed in a dorsal recumbent position and a transverse incision was made just caudal to the xiphoid process. The abdomen and chest cavities were incised. The diaphragm was incised, followed by the left and right lateral ribcage. The xiphoid process was clamped and pulled rostrally to hold the chest cavity open. An 18-gauge needle, connected to a two-way valve was then inserted into the left ventricle and the right auricle was incised. Transcardial perfusion was done in two stages. First, exsanguination was accomplished using phosphate buffered saline (PBS) solution, pH 7.3-7.5. Then, the tissues were fixed using 4% PBS-buffered Paraformaldehyde (4% PBS/PF) solution. Two-hundred milliliters of fixative were allowed to flow for 10-15 minutes.

Once perfusion was complete, the brain, 2 cm of the colon, jejunum, and ileum were collected and post-fixed by immersion in 4% PBS/PF solution for at least a week. The remainder of the colon was dissected out, cut along its longitudinal axis, and washed in PBS to remove fecal material. It was then stretched and pinned, mucosal side up, on a piece of Styrofoam™ and washed several more times in PBS. Finally, it was further fixed with 4% PBS/PF solution where it remained overnight at 4° C.

Tissue Preparation: Longitudinal Muscle-Myenteric Plexus Sections

On day following perfusion and fixation, the 4% PBS/PF solution was decanted from the stretched and pinned colon sections. They were then rinsed several times in PBS and cut with a scalpel into approximately one by one centimeter square sections. These sections were stored in 24-well plates in 0.1 M PBS.

The myenteric plexus of each section were dissected with the aide of fine forceps under a dissecting scope. Peeling consisted of first positioning the section so that the serosal surface was facing upward and the mucosal surface was in contact with the bottom of the petri dish. Then a corner of the section was gently pinched until the inner circular layer started to separate from the submucosal layer beneath or the myenteric plexus started to separate from the inner circular layer. These layers were gently pulled apart. The tissue was turned over so that the serosal surface faced the bottom of the petri dish. In most cases, inner circular muscle adhered to the myenteric plexus and had to be peeled away in strips.

Tissue Preparation: Paraffin-Embedded Sections

Following 4% PBS/PF fixation, colon cross-sections were rinsed twice in distilled water, placed in cassettes, and placed in 70% ethanol. Tissues were paraffin-infiltrated in a V.I.P. Tissue Tek Processor (Miles/Sakura, Torrance, CA). Tissues were then embedded in Paraplast X-Tra Tissue Embedding Medium (Fisher Scientific, Pittsburgh, PA) at a Tissue-Tek embedding station (Miles/Sakura, Torrance, CA). When processing was complete, tissues were removed from cassettes and embedded in a block of paraffin. Seven micron-thick sections were cut using a microtome (2040, Reichert-Jung, Depew, NY), floated onto positively-charged slides (Superfrost/Plus Precleaned Microscope Slides, Fisher Scientific, Pittsburgh, PA), and air-dried overnight.

Immunohistochemistry (IHC)

Immunofluorescence was used to observe protein expression tissues. The intensity of the signal is considered directly proportional to the amount of protein present, allowing amplified visualization of the antibody-antigen (target protein) complex. For paraffin embedded tissues, the following protocol was carried out: Slides were deparaffinized and rehydrated by washing twice in Hemo De (Scientific Safety Solvents), twice in absolute ethanol, once in 95% ethanol, once in 70% ethanol, and once in distilled water, for three minutes each. An antigen retrieval step was required for the GFAP antibody. The slides were boiled in 10mM sodium citrate (pH 6.0) for 20 minutes. When cool, slides were washed once with distilled water and washed in PBS three times three minutes each. Sections were then blocked for 20 minutes in normal blocker, which consisted of 5% normal serum from the same species in which the secondary antibody was raised and 2.5% bovine serum albumin (BSA) in PBS. Slides were rinsed in PBS, and incubated overnight in primary antibody diluted in normal blocker.

The next day, slides were washed three times in PBS three minutes each. The tissues were then incubated in fluorescent-conjugated secondary antibody for one hour in the dark. The slides were then washed twice in PBS in the dark for three minutes each. They were mounted in Vectashield (Vector Laboratories). The edges were sealed with clear nail polish and they were allowed to dry under aluminum foil for one hour and examined with a Nikon Eclipse E600 microscope equipped with epifluorescence.

## Reverse-Transcription Polymerase Chain Reaction

### Total RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen-Life Technologies Inc., Carlsbad, CA), according to the manufacturer's protocol. RNA concentrations were determined at 260 nm wavelength and the ratio of 260/280 was obtained using a UV spectrophotometer (DU640, Beckman Coulter Fullerton, CA). Samples with a 260/280 ratio of $\geq$ 1.8 were used.

### First strand synthesis (cDNA)

First strand synthesis was accomplished with a SABiosciences first strand kit C-03 (SABiosciences, Frederick, MD) according to the manufacturer's protocol. The kit incorporates a genomic DNA elimination step to prevent the amplification of contaminating genomic DNA in the RNA sample. Two micrograms of total RNA were used for each cDNA synthesis.

### Semi-quantitative and quantitative RT-PCR methods

Initially, semi-quantitative RT-PCR was used to determine the presence of melanocortin -1, -3, -4 and -5 receptors in rat colon using validated rat specific primer sets (SABiosciences) according to the manufacturer's protocol. RT-PCR was performed using Reaction Ready$^{TM}$ Hot Start''Sweet'' PCR Master Mix (SABiosciences). PCR products were analyzed in parallel with PCR products from the ribosomal S-15

housekeeping gene (361 bp, Ambion- AB Applied Biosystems Inc. Austin, TX, USA). Final end PCR products were viewed on 2% agarose gel.

Quantitive RT-PCR was used to determine the expression level of MC4R in treated and untreated groups using a method we described previously.[15] Briefly, reactions were performed in a 25 µl reaction mixture containing 12.5 µl RT2 Real-Time SYBR/Fluorescein Green PCR master mix with final concentrations of 10 mM Tris-Cl, 50 mM KCl, 2.0mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units of HotStart Taq DNA polymerase (SABiosciences), 1 µl first strand cDNA, 1 µl RT2 validated MC4R primer sets, and 10.5 µl PCR-grade water. Reactions were run in 96-well PCR plates using a PCR cycler (Bio-Rad, MyiQTM , Hercules, CA). All reactions were run in duplicate and the results were normalized to the GAPDH housekeeping-gene. The amplification protocol was set at $95^0$C for 15 min, and 40 cycles each at: $95^0$C for 30 s, $55^0$C for 30 s, and $72^0$C for 30 s.

Statistical Analysis

Analysis of quantitative RT-PCR data was performed using a modification of the delta delta *Ct* method ($\Delta\Delta Ct$) as described previously.[15, 63] $\Delta$ *Ct* for the MC4R gene in control cDNA samples (from untreated rats) and from MTII-treated rats was calculated by subtraction of the threshold cycle (CT) of the reference gene (GADPH) from the CT value of the target (MC4R) for normalization of CT values. Expression level (fold changes) were calculated using a formula of $2^{x-y}$, where *x* is the normalized CT value obtained from MTII-treated rats for each primer set and *y* is the normalized CT value obtained from control untreated rats.

## Results

Immunohistochemical techniques were used for labeling of MC4R, GFAP, and neuron-specific β-III tubulin in colon cross-sections and longitudinal muscle-myenteric plexus (LMMP) floating sections. Semi-quantitative and quantitative PCR were used to determine MC4R mRNA expression in the colon.

### Semi-quantitative RT-PCR analysis of MC4R in the gastrointestinal tract.

As a first step toward assessing the specific expression of MC4R in the colon, we identified MC4R mRNA expression in all of the segments of the gastrointestinal tract. We used RT-PCR on Trizol-homogenized whole tissue samples from the rat stomach (ST), duodenum (DU), jejunum (JE), ileum (IL), cecum (CE), and colon (CO). Because MC4R is predominantly expressed in the hypothalamus we used hypothalamic tissue as a positive control. An RNA sample of pooled isolated RNA from the experimental animals, before reverse-transcription, was used as a negative control to rule out the possibility of amplification of contaminating genomic DNA in our RNA samples. No band was detected in the negative control. Overall, RT-PCR analysis showed the presence of MC4R in all gastrointestinal sections (Fig. 7).

**Semi-quantitative RT-PCR analysis of MCR subtypes -1, -3, -4, and -5 in the colon.**

We asked which MCR subtypes (MC1R and MC3R-MC5R) that are receptors for α-MSH are expressed in the colon. Semi-quantitative RT-PCR analysis showed expression of mRNA of all the receptors analyzed. (Fig.8). MC2R was excluded from the analysis because it is a receptor for ACTH and not α-MSH. An RNA sample of pooled isolated RNA from the experimental animals, before reverse-transcription, was used as a negative control to rule out the possibility of amplification of genomic DNA in our RNA samples.

**Immunohistochemical localization of MC4R in the colon myenteric plexus.**

We used IHC to determine which cells types in the colon expressed MC4R in longitudinal muscle-myenteric plexus (LMMP) floating sections and paraffin-embedded cross-sections to. Myenteric neurons in the colon were immunoreactive for MC4R (Fig. 9)**.** To further confirm that neurons were expressing MC4R, we ran co-localization assays using anti-neuron-specific beta III tubulin and anti-MC4R antibodies. (Fig. 10) Beta-tubulin was labeled with a red fluorophore and MC4R with a green fluorophore. A yellow color was present, which is indicative of co-localization.

**Immunohistochemical localization of MC4R in colon EGCs.**

To examine the anti-inflammatory potential of MC4R in the colon, we used IHC to see whether or not MC4R is expressed by EGCs in the colon. We used anti-GFAP and

anti-MC4R antibodies in a co-localization assay to localize MC4R to EGCs in the colon (Fig. 11). MC4R was labeled with a red fluorophore and GFAP was labeled with a green fluorophore. The yellow color surrounding the neuronal cell bodies suggests that the ECGs which surround the neurons express MC4R as well as GFAP.

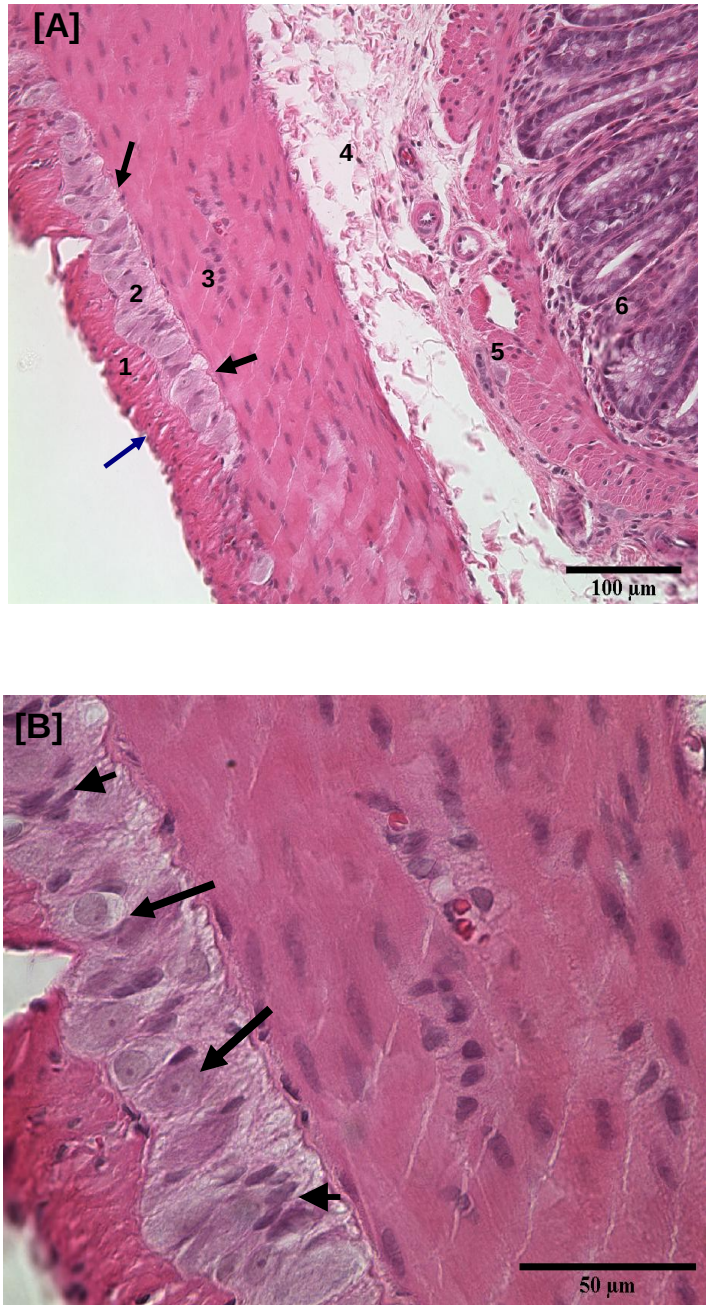
**Immunohistochemical comparison of the effect of icv and peripheral injection of MTII, leptin, or vehicle on MC4R expression in the colon myenteric plexus.**

We carried out an in vivo experiment to examine the effect of the MC4R agonist MTII on MC4R expression in the colon. An IHC comparison of our treatment groups indicated that there was no visible difference between the effect of icv and peripheral injection of MTII, icv injection of leptin, or icv and peripheral injection of vehicle on MC4R intensity in the colon myenteric plexus (Fig. 12). The intensity of signal of the control groups was comparable to that of the MTII treated groups and greater than that of the leptin group. All sections shown were from the same assay and all images were captured at the same exposure. MC4R is labeled with a green fluorophore.

**Quantitative RT-PCR comparison of the effect of MTII or vehicle icv injection on MC4R expression in the colon.**

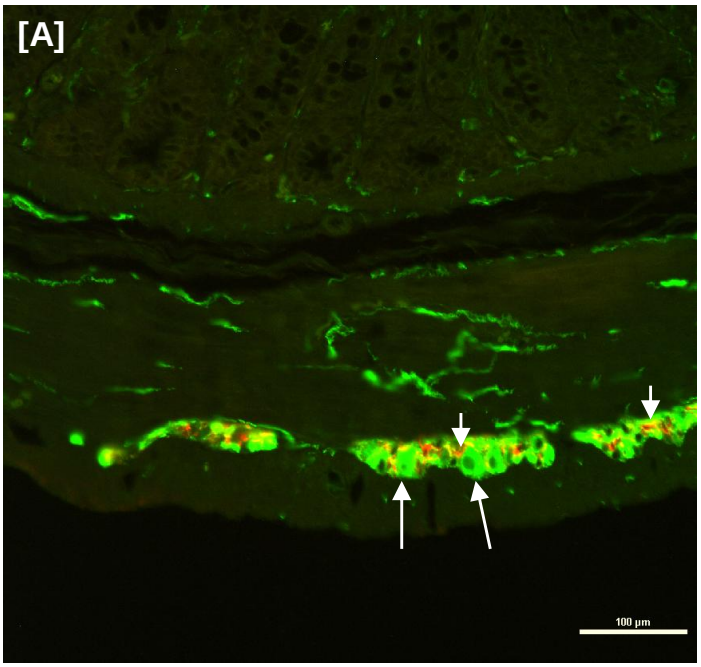
Similar to the IHC data, our real-time PCR data showed no significant differences in MC4R expression in the MTII group compared to the vehicle group (Fig. 13). The Y-axis has relative expression levels in four individuals from each group (X-axis).

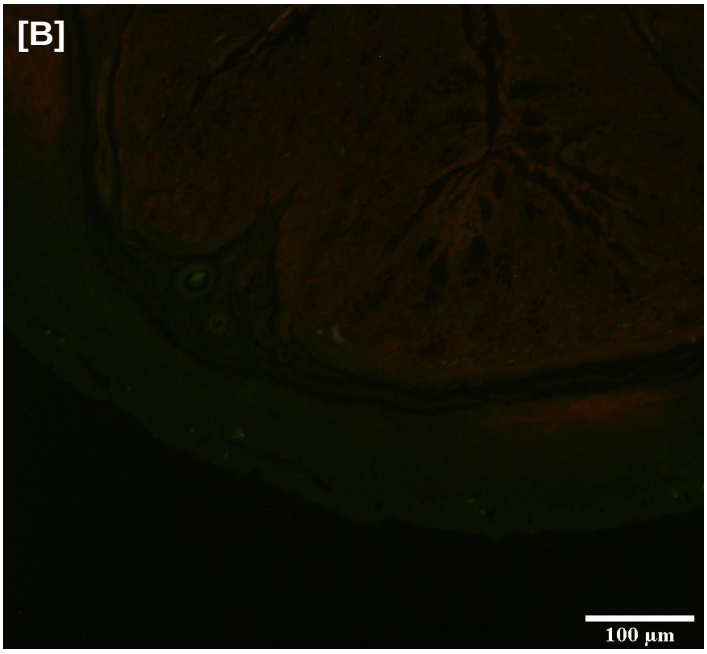
**Fig. 4. H&E Stained Paraffin-Embedded Rat Colon Cross-Section**




H&E stained paraffin-embedded colon section, illustrating the location and morphology of the myenteric plexus at **[A]** 20X and **[B]** 60X. **[A]** shows all of the layers of the colon, from left to right: serosal surface (blue arrow), outer longitudinal muscle (1), myenteric plexus (2), inner circular layer (3), submucosa (4) and submucosal plexus (5), and the mucosal layer (6). The lumen (not shown) is to the right. The myenteric plexus is located between the outer longitudinal layer and the inner circular layer. **[B]** The black arrows are pointing to the myenteric neurons and the arrowheads are pointing to the enteric glial cells (EGCs). Note that the EGCs largely outnumber the neurons.

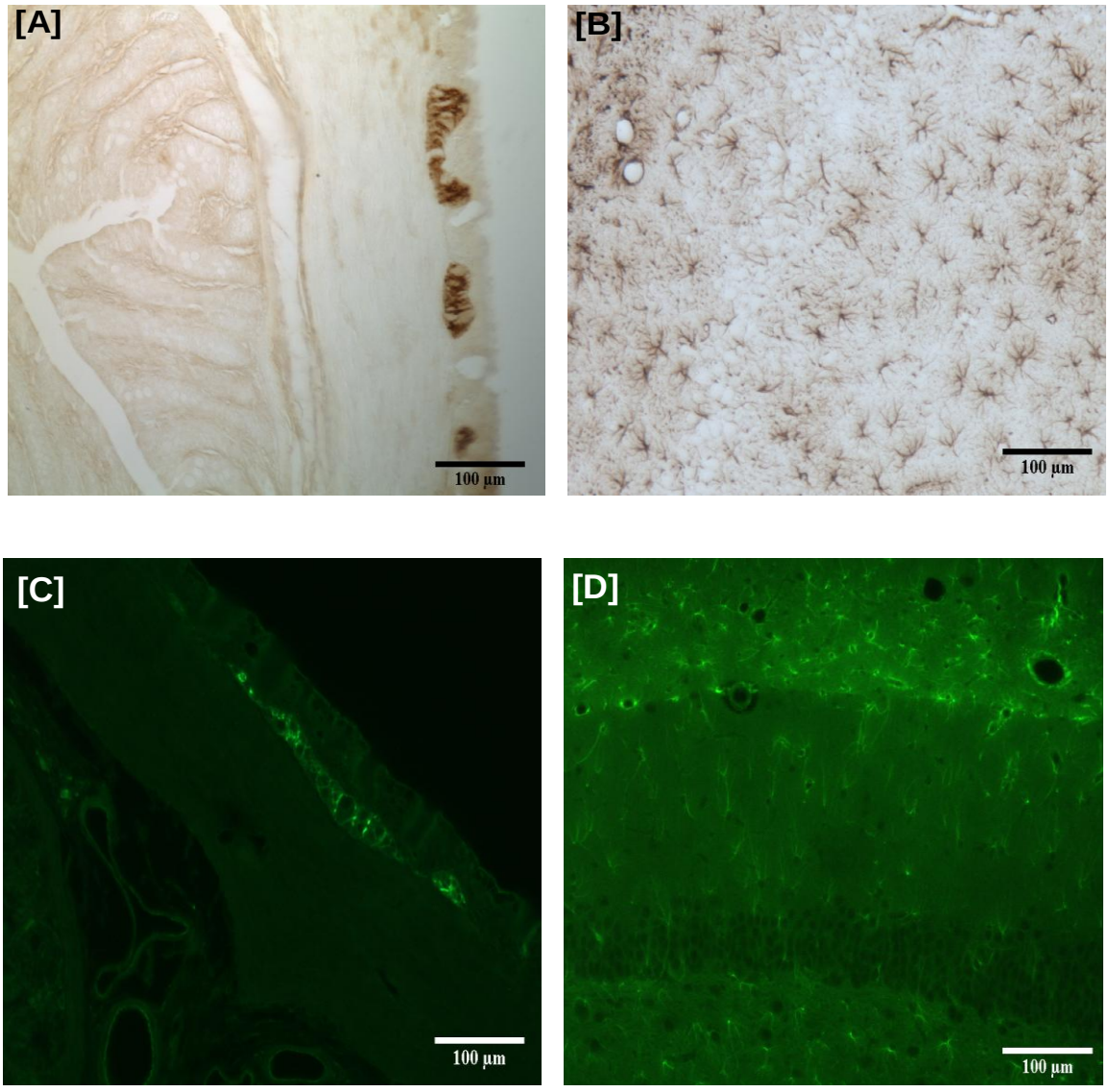
**Fig. 5. Immunofluorescent Labeling of Myenteric Neurons and Enteric Glial Cells in the Rat Colon**



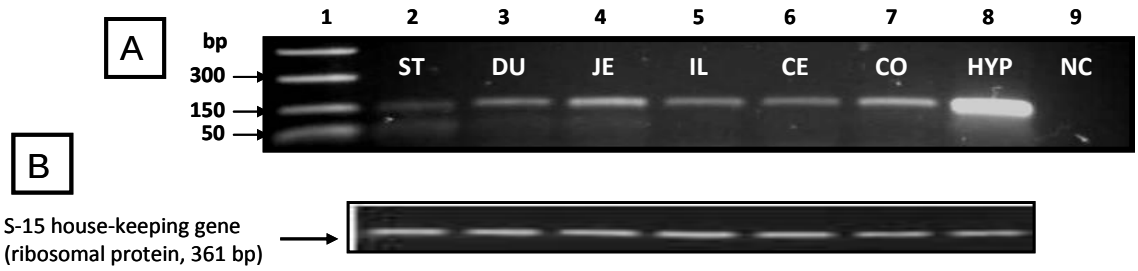

Representative micrograph showing **[A]** immunoreactivity of myenteric neurons (white arrows) for neuron-specific β-III tubulin (green) and enteric glial cells (white arrowheads) for GFAP (red) in the colon. 20X. **[B]** Negative control. 20X.

## Fig. 6. GFAP is a Marker for Enteric Glial Cells in the Colon and Astrocytes in the Brain



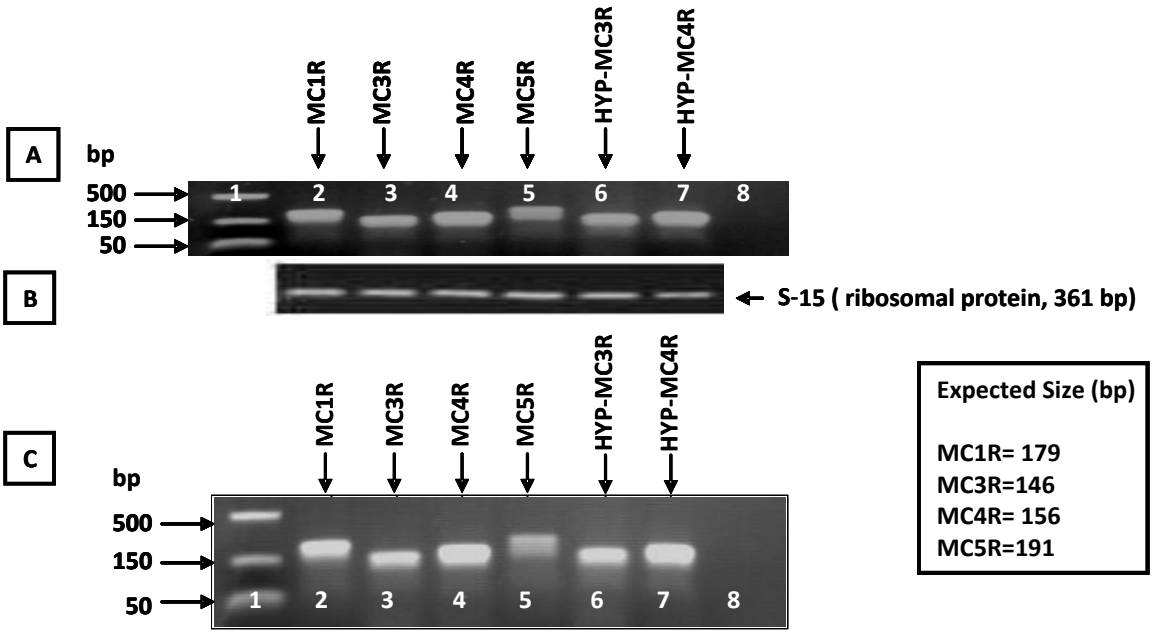

Representative micrographs showing DAB **[A&B]** and immunofluorescent labeling **[C&D]** of GFAP in enteric glial cells in the colon **[A&C]** and astrocytes in the brain **[B&D].** GFAP is a marker for both populations of glial cells.

## Fig. 7. Semi-Quantitative RT-PCR Detection of MC4R in the Rat Gastrointestinal Tract and Hypothalamus



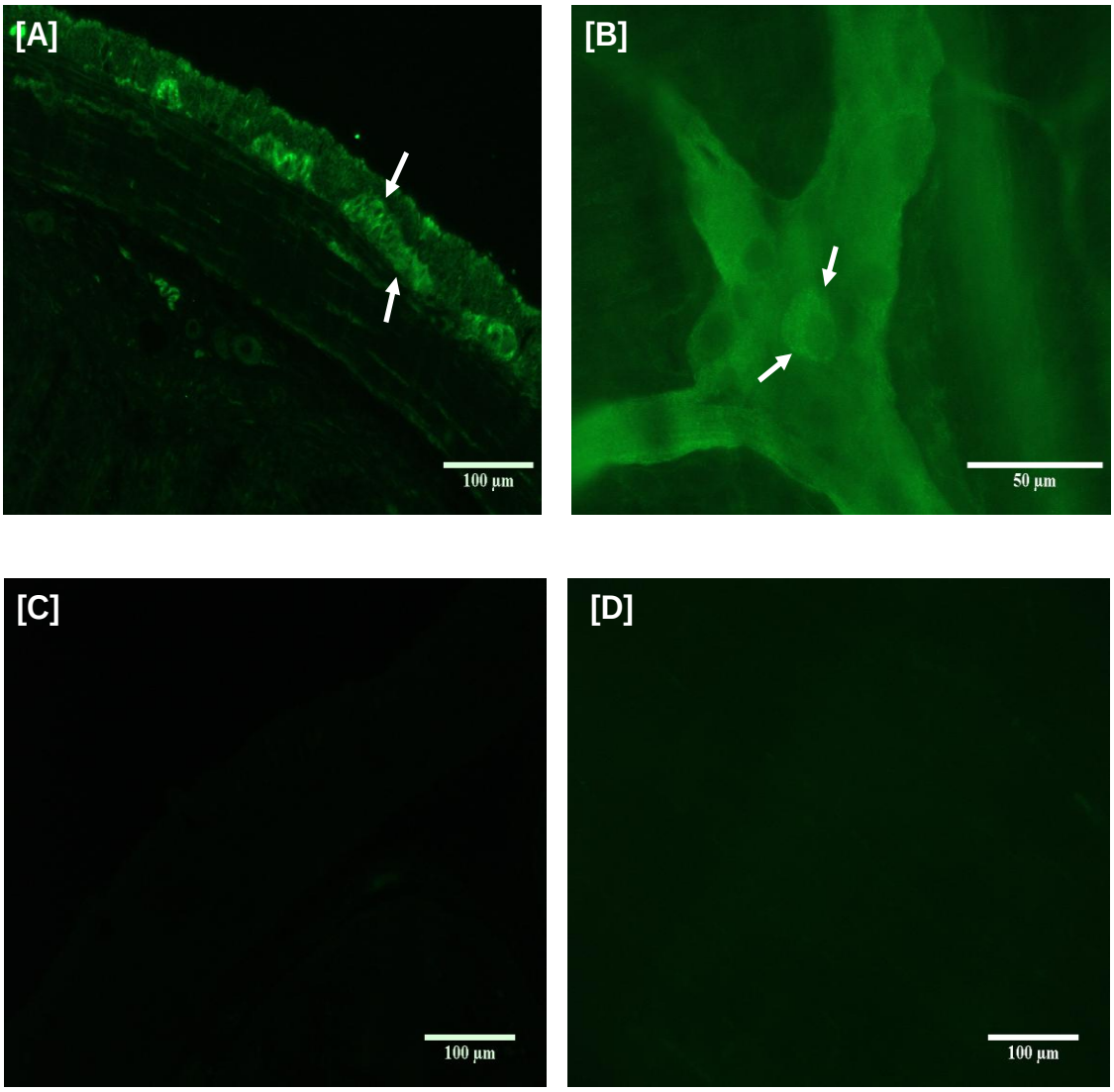

**[A]** 2% agarose gel showing mRNA expression of MC4R (156 bp) in various segments of the rat gastrointestinal tract and the hypothalamus. ST = stomach, DU =duodenum, JE = Jejunum, IL = Ileum, CE = cecum, and CO = colon, in lanes 2-7, respectively. Hypothalamic tissue (HYP) was used as a positive control in lane 8. Total RNA was loaded in lane 9 as a negative control (NC). **[B]** S-15 housekeeping gene (ribosomal mRNA) was amplified in parallel from samples used in **[A]** to verify the integrity of cDNA.

**Fig. 8. Semi-Quantitative RT-PCR Detection of MCR -1, - 3, - 4, and - 5 Subtypes in the Rat Colon**



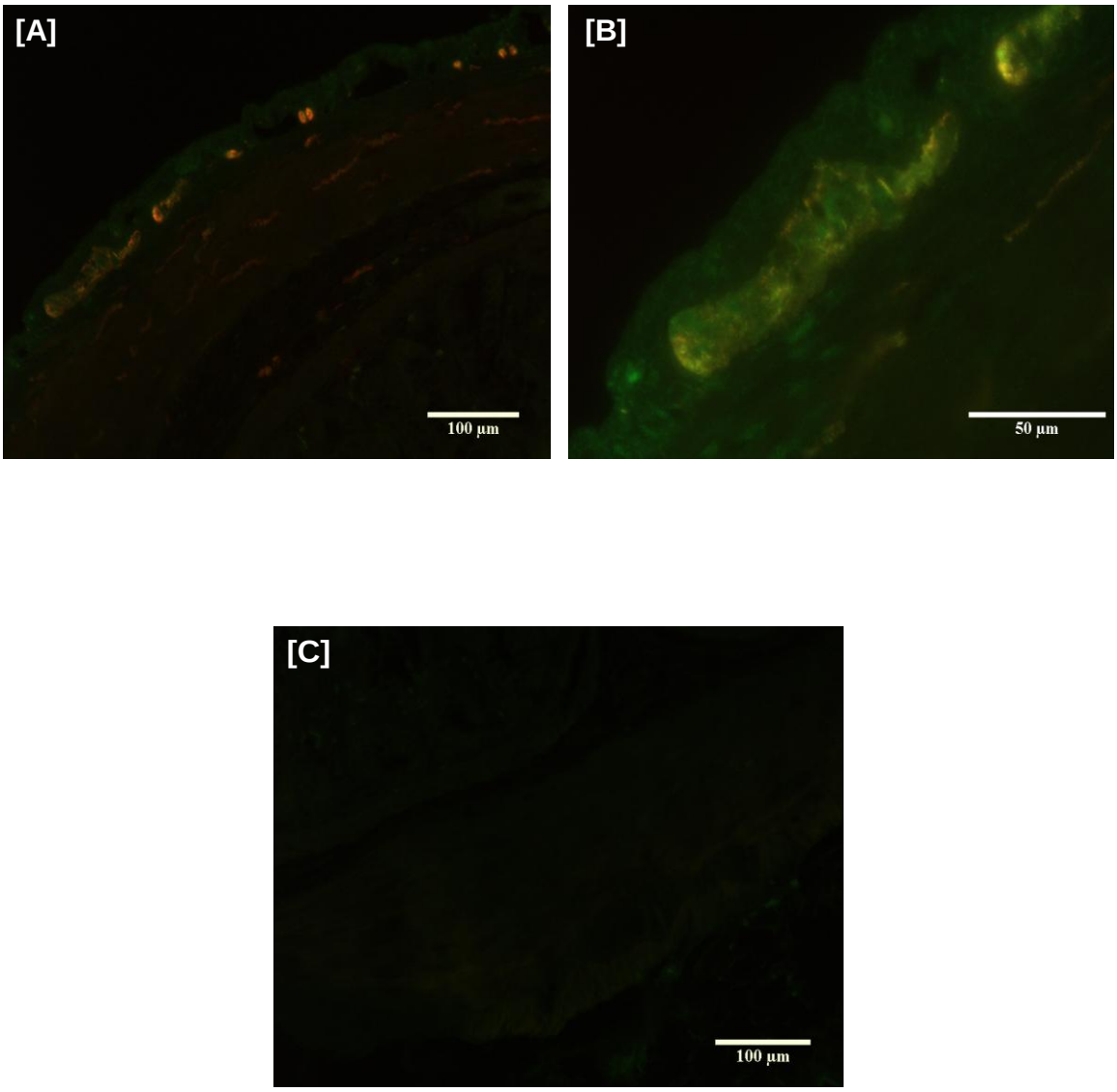

**[A]** 2% agarose gel showing mRNA expression of melanocortin -1, -3, -4, and -5 receptors in the rat colon (lanes 2-5, respectively). MC2R is excluded because it binds ACTH, not α-MSH. Lanes 6 and 7 show HYP-MC3R and HYP-MC4R (hypothalamic MC3R and MC4R) included as positive controls. Total RNA was loaded in lane 8 as a negative control. **[B]** S-15 housekeeping gene (ribosomal mRNA) amplified in parallel from samples used in **[A]** to verify the integrity of the cDNA. **[C]** Positive control cDNA samples were run in parallel from MC1R (skin), MC3R and MC4R (hypothalamus), and MC5R (prostate) to verify the expected product size for primer pairs used in **[A]**. Each band represents a pool of four cDNA samples from normal rats.

**Fig. 9. Immunohistochemical Localization of MC4R in the Colon Myenteric Plexus**



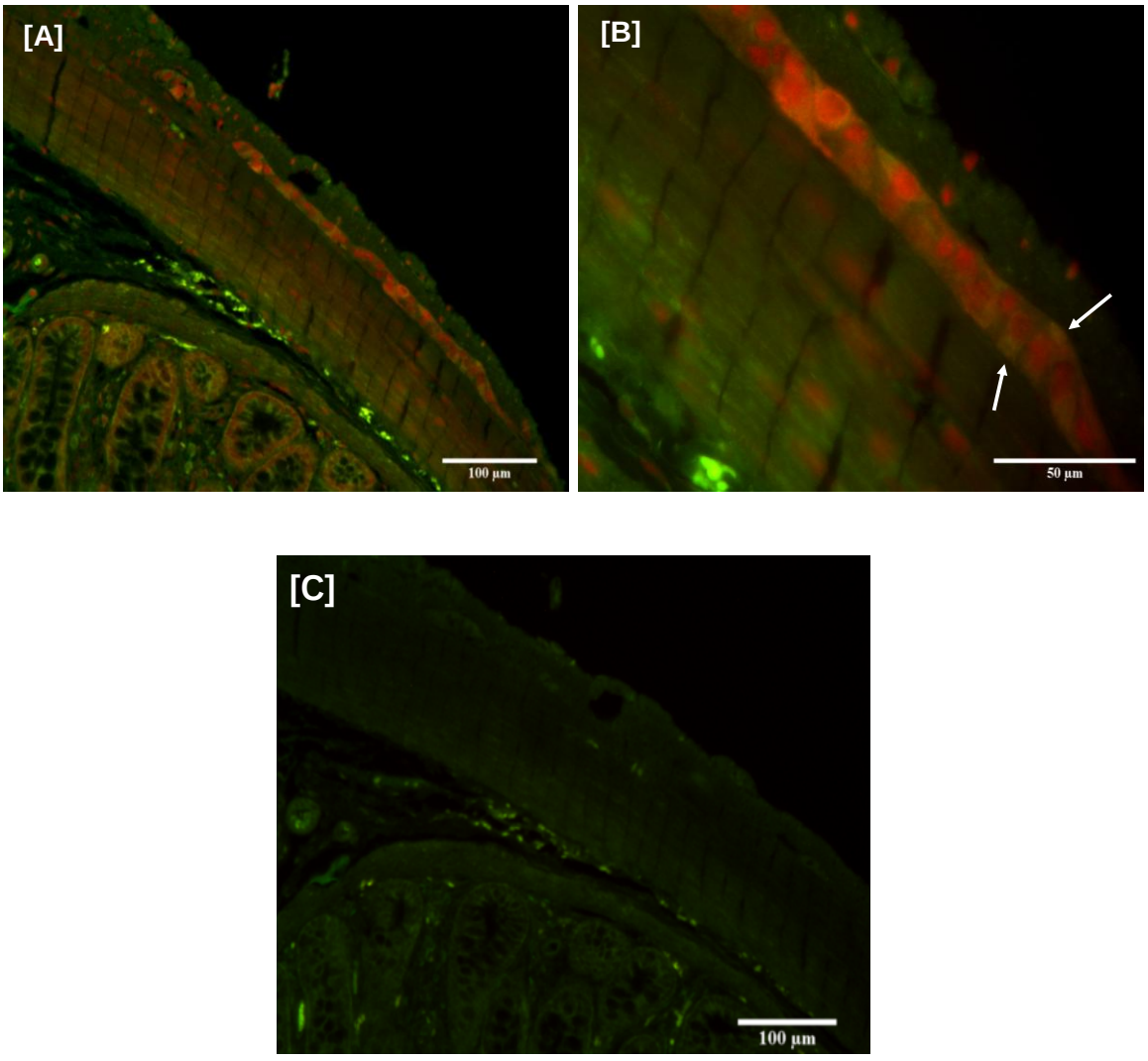

Representative micrographs showing immunoreactivity of myenteric neurons for MC4R. **[A]** Paraffin-embedded section, 20X. **[B]** Longitudinal Muscle-Myenteric Plexus (LMMP) floating section, 60X. **[C]** Negative control paraffin-embedded section, 20X. **[D]** Negative control LMMP section, 20X. Note staining around the periphery of the neuronal cell bodies (white arrows).

**Fig. 10. Co-localization of Beta III Tubulin and MC4R in the Colon Myenteric Plexus**



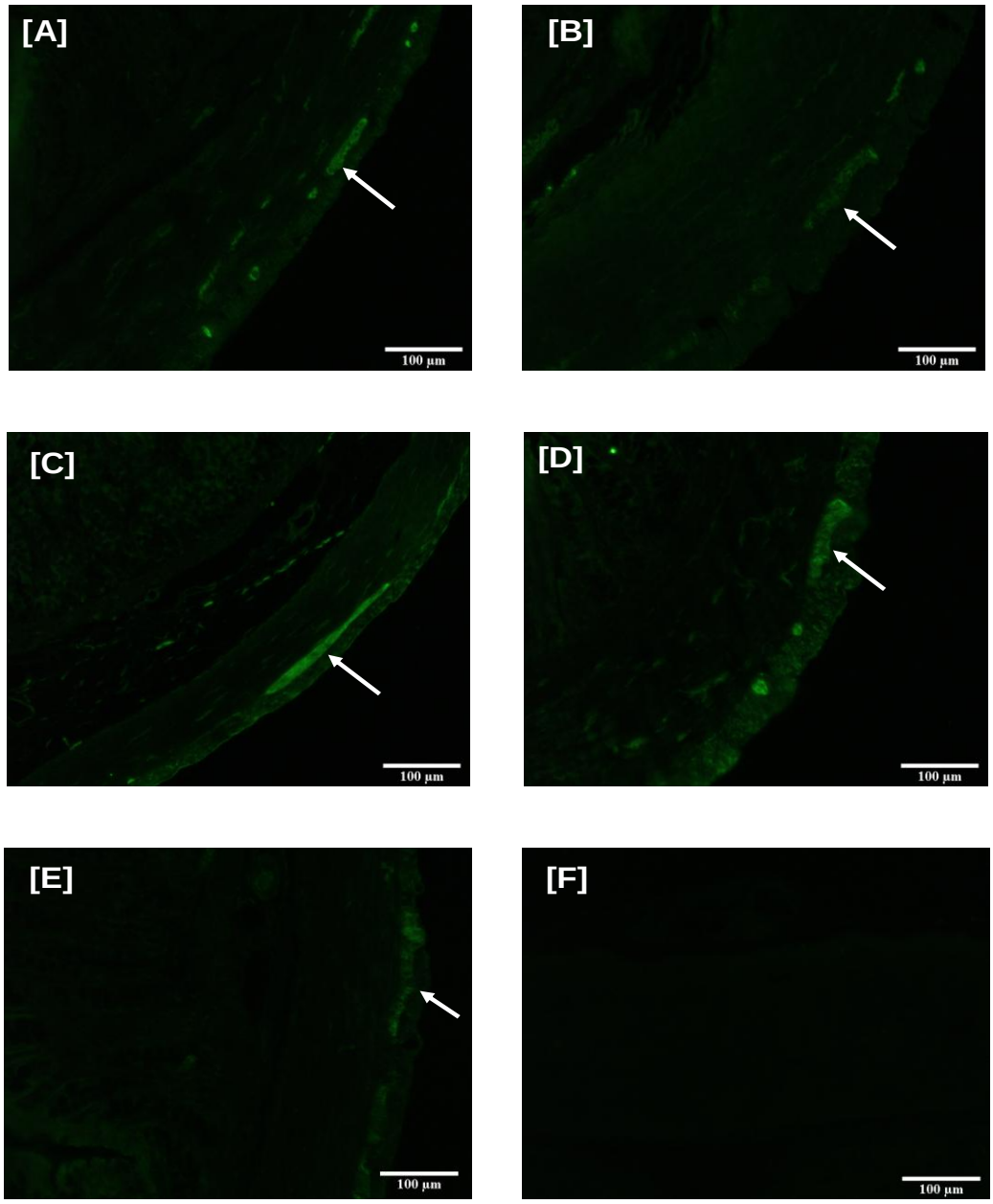

Representative micrographs showing immunoreactivity of myenteric neurons for neuron-specific β-III tubulin (red) and MC4R (green) in the colon at **[A]** 20X and **[B]** 60X. **[C]** is a negative control at 20X. The yellow fluorescence of the neurons implies that they express MC4R as well as neuron-specific β-III tubulin.

**Fig. 11. Co-localization of MC4R and GFAP in Colon Enteric Glial Cells**



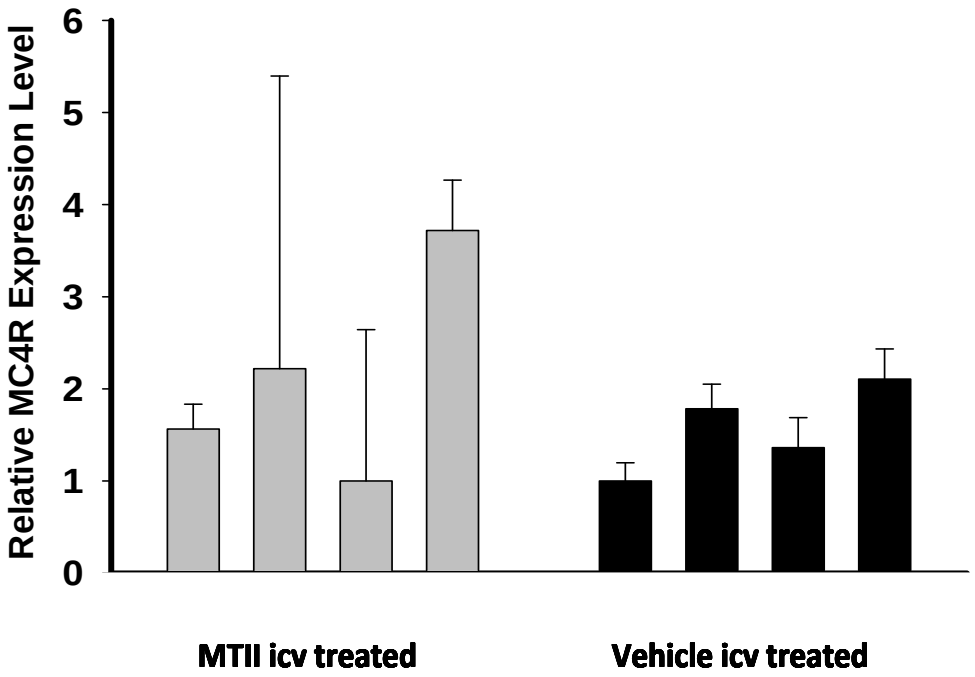

Representative micrographs showing immunoreactivity of enteric glial cells (EGCs) for MC4R(red) and GFAP (green) in the colon at **[A]** 20X and **[B]** 60X. **[C]** is a negative control taken at 20X. The MC4R-expressing myenteric neurons fluoresce red and the EGCs fluoresce yellow (white arrow), suggesting that they express GFAP and MC4R.

## Fig. 12. Immunohistochemical Comparison of MC4R Expression among MTII, Leptin, and Vehicle Groups




Representative micrographs showing MC4R intensity 90 min. after a single dose of MTII. Leptin, or vehicle. **[A]:** Group 1, 50 ng MTII, icv; **[B]:** Group 2, 5 µg Leptin, ip; **[C]:** Group 3, 50ng MTII, ip; **[D]:** Group 4, vehicle, icv; **[E]:** Group 5, Vehicle, ip; **[F]:** Negative Control. Staining intensities of the treatment groups do not appear greater than the control groups.

**Fig. 13. Quantitative RT-PCR Analysis of MC4R Expression Level in Treated vs. Control ICV Groups**




The difference in MC4R expression in the MTII group compared to the vehicle group was not statistically significant. On the Y-axis are the relative MC4R expression levels in four individuals from each group (X-axis).

## Discussion

In this study, we showed that MC4R is expressed in colon myenteric neurons and EGCs. Semi-quantitative RT-PCR and IHC analysis showed that MC4R mRNA and protein, respectively, are expressed in the colon. Semi-quantitative RT-PCR showed that MC4R mRNA is expressed in other segments of the gastrointestinal tract and that mRNA of MCRs -1, -3, and -5 are also expressed in the colon. Using IHC for further analysis, we localized MC4R to the myenteric neurons and surrounding EGCs. These findings suggest physiological involvement of MC4R in the functions of the colon myenteric plexus and EGCs.

Following the establishment of MC4R presence in the colon ENS, we wanted to know what effect treatment with MTII, a synthetic α-MSH analog, had on the expression of MC4R in the myenteric plexus and EGCs. We hypothesized that a single 50 ng dose (icv or ip) of MTII would stimulate upregulation of MC4R mRNA and protein. We used different routes of administration to determine whether or not there would be a difference in MC4R expression stimulated by a centrally or peripherally administered ligand. We also hypothesized that a single 5μg dose (icv) of leptin might have a similar effect because leptin stimulates the release of α-MSH.[16] As shown by quantitative RT-PCR and IHC, there was no significant increase in the expression level of MC4R mRNA or MC4R signal intensity in MTII-treated and leptin-treated groups compared to untreated (vehicle) groups.

There are several possibilities as to why there was no significant difference in MC4R intensity between the MTII, leptin, and vehicle treatment groups. A single dose may not have been enough to stimulate upregulation of MC4R. The effect may have been greater if there were a chronic administration of MTII over a period of several days but this possibility would require further investigation.

Another limitation to our study is that MTII is an agonist for both MC3R and MC4R .[64] Therefore, it is possible that the effect of MTII on cells expressing MC4R was diminished by MTII binding to MC3R, reducing the concentration of the ligand in circulation that could bind MC4R. Our semi-quantitative RT-PCR results detected MC3R mRNA in the colon but we have examined protein expression nor localized it to a specific cell type. MTII may also have bound MC3R in the CNS. Because leptin stimulates POMC neurons in the arcuate nucleus to release α-MSH which binds both MC3R and MC4R, leptin treatment may have also lead to non-specific agonist binding of MC3R .[16] One way to circumvent non-specific binding to receptors other than MC4R in future studies would be to use an MC4R-specific agonist such as tetrahydroisoquinoline (THIQ), which is over 500-fold more selective for MC4R than other MCRs in binding assays.[65]

Yet another possible explanation for the failure of MTII to induce significant upregulation of MC4R in our treated groups is agonist-mediated desensitization and internalization of MC4R. Desensitization is a process that occurs in many G protein-coupled seven transmembrane receptors in which signaling is quickly diminished within minutes of exposure to an agonist.[66] It is associated with phosphorylation of serine/threonine residues of the G protein-coupled receptor by G protein-coupled receptor

kinases (GRKs). Phosphorylation allows the G protein-coupled receptors to interact with cytoplasmic proteins called arrestins that sterically block the receptors from G-proteins. The G protein-coupled receptors are then sequestered (or internalized) into clathrin-coated intracellular vesicles that have pinched off from the plasma membrane. Following internalization, the G protein-coupled receptors may either be recycled to the cell membrane or be subject to lysosomal degradation. [66, 67]

Previous work has shown that MC4R exhibits agonist-mediated desensitization. One study showed that pretreatment with 100 nM α-MSH for 10-180 minutes caused an exposure time-dependent decrease of cAMP formation by a second equimolar α-MSH treatment. The desensitization occurred rapidly by 30 minutes and progressively increased up to 180 minutes.[66] Our animals were euthanized 90 minutes post-treatment, which is an appropriate time-frame for desensitization to take place. One way to circumvent this problem in future studies would be to euthanize the animals less than 30 minutes post-treatment.

Our IHC data supports the hypothesis that MC4R is expressed in colon myenteric neurons and EGCs. IHC detection of MC4R in colon EGCs is a very interesting finding that could have important clinical implications as discussed in the following points. First, the detection of MC4R in colonic EGCs is reminiscent of previous studies showing MC4R expression in astrocytes in the brain. [58] As astrocytes represent an important neuroprotective defense against inflammation in the brain, a similar role could be postulated for the EGCs of the colon. [58] Several studies have shown the importance of these cells in protecting neurons from inflammatory insults. Loss of enteric glia in animal models causes neuronal degeneration, which is characteristic of inflammation in the

gastrointestinal tract .[52, 68] Second, activation of MC4R via α-MSH has potent anti-inflammatory, antipyretic, and antimicrobial actions. [59, 60, 69] These protective effects could be exploited in the colon clinically because of susceptibility of the colon to inflammatory diseases such as IBD. Third, α-MSH has been shown to suppress inflammatory responses via repression of NF-κB.[12] In astrocytes, NF-κB activation results in the production of pro-inflammatory mediators including the pro-inflammatory cytokines TNF-α, IL-1, and IL-6.[70] These cytokines are expressed in IBD and have been attributed to immune cells but may also be products of EGCs.[43, 48, 71] Agents, such as α-MSH, that prevent the activation of effects of NF-κB can inhibit colonic inflammatory diseases.

In summary, an MC4R-specific agonist could be potentially useful in the treatment of neurodegenerative and inflammatory diseases of the colon by acting on MC4R receptors in colon EGCs to reduce the production of pro-inflammatory cytokines that exacerbate inflammation. Further studies to explore the role of MC4R in the myenteric plexus and EGCs of the colon are warranted.

## References


1. **Gautron L, Lee C, Funahashi H, Friedman J, Lee S, Elmquist J. Melanocortin-4 receptor expression in a vago-vagal circuitry involved in postprandial functions. J Comp Neurol 2010;518:6-24.**

2. **Cone RD. Studies on the Physiological Functions of the Melanocortin System Endocrine Reviews 2006;27:736-749.**

3. **Lasaga M, Debeljuk L, Durand D, Scimonelli TN, Caruso C. Role of alpha-melanocyte stimulating hormone and melanocortin 4 receptor in brain inflammation. Peptides 2008;29:1825-1835.**

4. **MacNeil DJ, Howard AD, Guan X, Fong TM, Nargund RP, Bednarek MA, Goulet MT, Weinberg DH, Strack AM, Marsh DJ, Chen HY, Shen CP, Chen AS, Rosenblum CI, MacNeil T, Tota M, MacIntyre ED, Van der Ploeg LH. The role of melanocortins in body weight regulation: opportunities for the treatment of obesity. Eur J Pharmacol 2002;450:93-109.**

5. **Gross KJ, Pothoulakis C. Role of Neuropeptides in Inflammatory Bowel Disease. Inflammatory Bowel Disease 2007;13:918-932.**

6. **Luger TA, Brzoska T. alpha-MSH related peptides: a new class of anti-inflammatory and immunomodulating drugs. Ann Rheum Dis 2007;66 Suppl 3:iii52-5.**

7. **Ni XP, Butler AA, Cone RD, Humphreys MH. Central receptors mediating the cardiovascular actions of melanocyte stimulating hormones. J Hypertens 2006;24:2239-46.**

8. **Tao YX. The Melanocortin-4 Receptor: Physiology, Pharmacology, and Pathophysiology. Endocrine Reviews 2010;31:1-38.**

9. **Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 1997;88:131-41.**

10. **Chen AS, Metzger JM, Trumbauer ME, Guan XM, Yu H, Frazier EG, Marsh DJ, Forrest MJ, Gopal-Truter S, Fisher J, Camacho RE, Strack AM, Mellin TN,**

**MacIntyre DE, Chen HY, Van der Ploeg LH. Role of the melanocortin-4 receptor in metabolic rate and food intake in mice. Transgenic Res 2000;9:145-54.**

**11. van der Kraan M, Adan RA, Entwistle ML, Gispen WH, Burbach JP, Tatro JB. Expression of melanocortin-5 receptor in secretory epithelia supports a functional role in exocrine and endocrine glands. Endocrinology 1998;139:2348-55.**

**12. Brzoska T, Luger TA, Maaser C, Abels C, Böhm M. α-Melanocyte-Stimulating Hormone and Related Tripeptides: Biochemistry, Antiinflammatory and Protective Effects in Vitro and in Vivo, and Future Perspectives for the Treatment of Immune-Mediated Inflammatory Diseases. Endocrine Reviews 2008;29:581-602.**

**13. Gantz I, Fong TM. The melanocortin system. Am J Physiol Endocrinol Metab 2003;284:E468-74.**

**14. Voisey J, Carroll L, van Daal A. Melanocortins and their Receptors and Antagonists. Current Drug Targets 2003;4:588-597.**

**15. Mansour M, White D, Wernette C, Dennis JC, Tao YX, Collins R, Parker LR, Morrison E. Pancreatic neuronal melanocortin-4 receptor modulates serum insulin levels independent of leptin receptor. Endocrine 2010;37:220-230.**

**16. da Silva AA, do Carmo JM, Freeman JN, Tallam LS, Hall JE. A Functional Melanocortin System May Be Required for Chronic CNS-Mediated Antidiabetic and Cardiovascular Actions of Leptin. Diabetes 2009;58:1749-1756.**

**17. Margetic S, Gazzola C, Pegg GG, Hill RA. Leptin: a review of its peripheral actions and interactions. Int J Obes Relat Metab Disord 2002;26:1407-33.**

**18. Cunningham DJ, Romanes GJ. Cunningham's Textbook of anatomy. Oxford University Press, 1981.**

**19. Dellmann H-D. Textbook of veterinary histology. Lea & Febiger, 1993.**

**20. Gartner LP, Hiatt JL. Color Textbook of Histology. W.B. Saunders Company, 2001.**

**21. Savidge TC, Sofroniew MV, Neunlist M. Starring roles for astroglia in barrier pathologies of gut and brain. Laboratory Investigation 2007;87:731-736.**

**22. Cabarrocas J, Savidge TC, Liblau RS. Role of Enteric Glial Cells in Cells in Inflammatory Bowel Disease. Glia 2003;41:81-93.**

**23. Goyal RK, Hirano I. The enteric nervous system. N Engl J Med 1996;334:1106-15.**

**24. Lomax AE, Fernandez E, Sharkey KA. Plasticity of the enteric nervous system during intestinal inflammation. Neurogastroenterol Motil 2005;17:4-15.**

**25. Gershon MD, Kirchgessner AL, Wade PR. Functional Anatomy of the Enteric Nervous System. In: Johnson LR, ed. Physiology of the Gastrointestinal Tract. Volume 1. New York: Raven Press, 1994:381-422.**

**26. Roskams AJ, Cai X, Ronnett GV. Expression of neuron-specific beta-III tubulin during olfactory neurogenesis in the embryonic and adult rat. Neuroscience 1998;83:191-200.**

**27. Gershon MD, Rothman TP. Enteric Glia. Glia 1991;4:195-204.**

**28. Neunlist M, Van Landeghem L, Bourreille A, Savidge T. Neuro-glial crosstalk in inflammatory bowel disease. J Intern Med 2008;263:577-83.**

**29. Ruhl A. Glial cells in the gut. Neurogastroenterol Motil 2005;17:777-90.**

**30. Squire RS, Bloom FE, McConnell SK, Roberts JL, Spitzer NC, Zigmoid MJ. Fundamental Neuroscience. Academic Press, 2003.**

**31. Miampamba M, Yang H, Sharkey KA, Tache Y. Intracisternal TRH analog induces Fos expression in gastric myenteric neurons and glia in conscious rats. Am J Physiol Gastrointest Liver Physiol 2001;280:G979-91.**

**32. Trowbridge HO, Emling RC. Inflammation : a review of the process. Quintessence Pub. Co., 1997.**

**33. Bernstein CN, Vidrich A. Isolation, identification, and culture of normal mouse colonic glia. Glia 1994;12:108-16.**

**34. Vasina V, Barbara G, Talamonti L, Stanghellini V, Corinaldesi R, Tonini M, De Ponti F, De Giorgio R. Enteric neuroplasticity evoked by inflammation. Auton Neurosci 2006;126-127:264-72.**

**35. Warner FJ, Liu L, Lubowski DZ, Burcher E. Circular muscle contraction, messenger signalling and localization of binding sites for neurokinin A in human sigmoid colon. Clin Exp Pharmacol Physiol 2000;27:928-33.**

**36. Oktar BK, Ercan F, Yegen BC, Alican I. The effect of alpha-melanocyte stimulating hormone on colonic inflammation in the rat. Peptides 2000;21:1271-7.**

**37. Rajora N, Boccoli G, Catania A, Lipton JM. α-MSH Modulates Experimental Inflammatory Bowel Disease. Peptides1997;18:381-385.**

**38. Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006;444:860-7.**

**39. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 2007;7:429-42.**

**40. Whitton PS. Inflammation as a causative factor in the aetiology of Parkinson's disease. British Journal of Pharmacology 2007;150:963–976.**

**41. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D,**

**Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. Inflammation and Alzheimer's disease. Neurobiol Aging 2000;21:383-421.**

**42. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115:182-205.**

**43. Abraham C, Cho JH. Inflammatory Bowel Disease. The New England Journal of Medicine 2009;361:2066-78.**

**44. Carter MJ, Lobo AJ, Travis SP. Guidelines for the management of inflammatory bowel disease in adults. Gut 2004;53 Suppl 5:V1-16.**

**45. Geboes K, Collins S. Structural abnormalities of the nervous system in Crohn's disease and ulcerative colitis. Neurogastroenterol Motil 1998;10:189-202.**

**46. Strober W, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. J Clin Invest 2007;117:514-21.**

**47. Gillen CD, Walmsley RS, Prior P, Andrews HA, Allan RN. Ulcerative colitis and Crohn's disease: a comparison of the colorectal cancer risk in extensive colitis. Gut 1994;35:1590-2.**

**48. Podolsky DK. Inflammatory Bowel Disease. New England Journal of Medicine 2002;347:417-429.**

**49. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol 2003;3:521-33.**

**50. van der Flier L, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. The Annual Review of Physiology 2009;71:241-60.**

**51. Crosnier C, Stamataki D, Lewis J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nat Rev Genet 2006;7:349-59.**

**52. Sanovic S, Lamb DP, Blennerhassett MG. Damage to the enteric nervous system in experimental colitis. Am J Pathol 1999;155:1051-7.**

**53. Neunlist M, Aubert P, Toquet C, Oreshkova T, Barouk J, Lehur PA, Schemann M, Galmiche JP. Changes in chemical coding of myenteric neurones in ulcerative colitis. Gut 2003;52:84-90.**

**54. Scheurer U, Drack E, Halter F. Substance P activates rat colonic motility via excitatory and inhibitory neural pathways and direct action on muscles. J Pharmacol Exp Ther 1994;271:7-13.**

**55. Jacobson K, McHugh K, Collins SM. Experimental Colitis Alters Myenteric Nerve Function at Inflamed and Noninflamed Sites in the Rat. Gastroenterology 1995;109:718-722.**

**56. Mullane KM, Kraemer R, Smith B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. J Pharmacol Methods 1985;14:157-67.**

**57. Catania A, Delgado R, Airaghi L, Cutuli M, Garofalo L, Carlin A, Demitri MT, Lipton JM. alpha-MSH in systemic inflammation. Central and peripheral actions. Ann N Y Acad Sci 1999;885:183-7.**

**58. Caruso C, Durand D, Schioth HB, Rey R, Seilicovich A, Lasaga M. Activation of melanocortin 4 receptors reduces the inflammatory response and prevents apoptosis induced by lipopolysaccharide and interferon-gamma in astrocytes. Endocrinology 2007;148:4918-26.**

**59. Sinha PS, Schioth HB, Tatro JB. Roles of melanocortin-4 receptor in antipyretic and hyperthermic actions of centrally administered alpha-MSH. Brain Research 2004;1001:150-158.**

**60. Catania A, Gatti S, Colombo G, Lipton JM. Targeting melanocortin receptors as a novel strategy to control inflammation. Pharmacol Rev 2004;56:1-29.**

**61. Coll AP. Effects of pro-opiomelanocortin (POMC) on food intake and body weight: mechanisms and therapeutic potential? Clin Sci (Lond) 2007;113:171-82.**

**62. Bush TG, Savidge TC, Freeman TC, Cox HJ, Campbell EA, Mucke L, Johnson MH, Sofroniew MV. Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. Cell 1998;93:189-201.**

**63. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:1-12.**

**64. Benoit SC, Schwartz MW, Lachey JL, Hagan MM, Rushing PA, Blake KA, Yagaloff KA, Kurylko G, Franco L, Danhoo W, Seeley RJ. A novel selective melanocortin-4 receptor agonist reduces food intake in rats and mice without producing aversive consequences. J Neurosci 2000;20:3442-8.**

**65. Martin WJ, MacIntyre DE. Melanocortin receptors and erectile function. Eur Urol 2004;45:706-13.**

**66. Shinyama H, Masuzaki H, Fang H, Flier J. Regulation of melanocortin-4 receptor signaling: agonist-mediated desensitization and internalization. Endocrinology 2003;144:1301-1314.**

**67. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. Journal of Cell Science 2002;115:455-465.**

**68. Bassotti G, Villanacci V, Fisogni S, Rossi E, Baronio P, Clerici C, Maurer CA, Cathomas G, Antonelli E. Enteric glial cells and their role in gastrointestinal motor abnormalities: Introducing the neuro-gliopathies. World Journal of Gastroenterology 2007;13:4035-4041.**

**69. Starowicz K, Przewlocka B. The role of melanocortins and their receptors in inflammatory processes, nerve regeneration and nociception. Life Sci 2003;73:823-47.**

**70. Memet S. NF-KB functions in the nervous system: From development to disease. Biochemical Pharmacology 2006;72:1180-1195.**

**71. Rogler G, Andus T. Cytokines in inflammatory bowel disease. World J Surg 1998;22:382-9.**