*Original Research* *Highlight article*

# The M1 muscarinic acetylcholine receptor in the crypt stem cell compartment mediates intestinal mucosal growth

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Impact statement

Localization of a specific subtype of the muscarinic acetylcholine receptor in the crypt stem cell compartment suggests a critical role in intestinal mucosal homeostasis. Here we demonstrate the localization of the M1 muscarinic acetylcholine receptor to the stem cell compartment and demonstrate increase morphometric and proliferative parameters when this is stimulated *in vivo.* These data provide novel information about this complex signaling microenvironment and offer potential future therapeutic targets for future study.

## Abstract

Maintenance of the highly plastic intestinal epithelium relies upon stem cells localized to intestinal crypts. Recent evidence suggests muscarinic acetylcholine signaling impacts epithelial barrier function, proliferation, and apoptosis. We hypothesized that the intestinal crypt base would express specific muscarinic acetylcholine receptors that drive proliferation in this critical region. Intestinal segments spanning the small bowel were procured from wild-type C57Bl/6 mice to determine muscarinic acetylcholine receptor mRNA expression and create sections on laser capture microdissection slides for analysis of crypt base cells. RT-PCR was performed using primers targeting the five muscarinic acetylcholine receptor subtypes (M1–M5), LGR5, BIII-tubulin, and GAPDH. To determine the effects of muscarinic agonism *in vivo*, osmotic pumps delivering the M1 muscarinic acetylcholine receptor agonist McN-A-343 were implanted into wild type mice for one week. Segments were harvested, histologic sections created, and morphometric and proliferative parameters measured. In full-thickness intestinal samples, muscarinic acetylcholine receptor subtypes M1–M4 were found in all regions, while M5 was localized to the proximal jejunum. In crypt-base cells, the M1 muscarinic acetylcholine receptor subtype was the only subtype found and was present in all regions. LGR5 was present in all laser capture microdissection samples, indicating the capture of intestinal stem cells. *In vivo* experiments conducted with McN-A-343 revealed significantly increased villus height, crypt depth, and crypt-cell proliferation. The presence of M1 muscarinic acetylcholine receptor mRNA within the stem cell niche in the intestinal crypt base coupled with increased mucosal growth with M1 receptor stimulation *in vivo* suggests that the cholinergic system, via the M1 muscarinic acetylcholine receptor, is a critical mediator of intestinal mucosal homeostasis.

**Keywords:** Muscarinic acetylcholine receptors, intestinal stem cells, mouse model, intestinal homeostasis, mucosal growth, intestinal crypts

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## Introduction

Maintenance of the small intestinal epithelium is a dynamic process which is essential for normal function and survival. The current understanding of intestinal epithelial cell homeostasis is based upon the paradigm of an intestinal stem cell niche located within the crypt base which gives rise to all epithelial cell types.[1,2] While the role of the enteric nervous system (ENS) and muscarinic acetylcholine (ACh) receptor signaling has been implicated in a broad range of epithelial functions and intestinal disease,[3–6] the mechanisms and interactions between intestinal stem cells and the various signaling cascades remain incompletely understood.

Previous data from our lab have suggested a role for muscarinic acetylcholine signaling in intestinal mucosal growth,[7] which stimulated our current investigations into the mechanisms by which this could occur. Given the known location of intestinal stem cells in the crypt base, we hypothesized that the intestinal crypt base would express only specific muscarinic ACH receptors

(mAChRs) that are critical in regulation of epithelial proliferation and homeostasis. Currently, few data exist characterizing this microenvironment with respect to mAChRs, and these studies are limited to localization of mAChR via immunofluorescence and have had conflicting results.[8,9] We aimed to specifically determine the expression of mAChR mRNA in the intestinal crypt stem cell compartment via RT-PCR, using laser capture microdissection (LCM) to accurately extract the crypt-base intestinal stem cell region for analysis. This approach allowed for a more precise analysis of the presence of mAChRs located specifically in the stem cell niche, and thus helped tailor our *in vivo* studies to only the receptor(s) present. We found that all subtypes of mAChRs are widely expressed in whole intestinal samples, but that the M1 subtype is uniquely expressed in the intestinal stem cell niche. Furthermore, stimulation of M1 mAChR *in vivo* results in increased mucosal growth, suggesting M1 is a critical mediator of intestinal mucosal homeostasis in the murine intestine. Further investigations into this mechanism are warranted in order to characterize the complex microenvironment involved in intestinal stem cell signaling.

## Materials and methods

### Animals

Wild type (WT) C57Bl/6 mice were bred at the Yale Animal Resource Facility; 12–14-week-old male mice were used for experiments. All animals were housed in a pathogen-free environment with a 12-h light/dark cycle with food and water ad libitum. All animal protocols were approved by Yale University's Institutional Animal Care and Use Committee.

### Whole intestinal sample preparation

Following $CO_2$ asphyxiation, a midline laparotomy was performed to harvest 2 cm segments from the proximal jejunum, distal jejunum, proximal ileum, and distal ileum of each mouse. Samples were then preserved overnight at 4°C in RNAlater solution (Thermo Fisher, Waltham, MA) per manufacturer recommendations. For these samples, sections were then placed onto specialized PEN membrane glass slides (Thermo Fisher) and stained with Nuclear Fast Red (Sigma, St. Louis, MO) per manufacturer protocols.

### Laser capture microdissection sample preparation

Segments (2 cm) were harvested as above from the proximal jejunum, distal jejunum, proximal ileum, and distal ileum of each mouse. Segments were fixed overnight in 10% neutral-buffered formalin. Sections were then placed onto specialized PEN membrane glass slides (Thermo Fisher) and stained with Nuclear Fast Red (Sigma, St. Louis, MO) per manufacturer protocols. Slides were placed on a laser microdissection microscope (Leica Microsystems, Wetzlar, Germany) and crypt bases visualized at 100× magnification. The target area was outlined and included the entire crypt base up to the approximate midpoint of the crypt. The laser was then initiated and the microdissected area of tissue was captured in RNAlater solution. At least five crypt base dissections were collected per slide, for a total of at least 10 dissections per sample.

### RNA extraction and RT-PCR

Both whole intestinal and LCM samples preserved in RNAlater were prepared and underwent RNA extraction according to the RNeasy Plus Mini Kit protocol (Qiagen, Germantown, MD). RNA extraction was also performed on WT whole brain tissue for use as a positive control for all five mAChRs. Following extraction, RT-PCR was performed in a one-step method using the OneStep RT-PCR Kit (Qiagen), using specific primers for all five mAChR subtypes. All RT-PCR experiments were performed on ice, and the following RT-PCR protocol was used: reverse transcriptase – 30 min at 50°C, PCR activation – 15 min at 95°C followed by 30 cycles of: 30 sec at 94 °C, 30 sec at 64°C, 1 min at 72°C, and final extension – 10 min at 72°C. To aid in determination of the ability of LCM to generate samples that included the intestinal crypt base stem cells but excluded surrounding neural tissues, we also amplified markers for crypt stem cells (LGR5), neuronal tissue (Beta-III tubulin), and a housekeeping gene (GAPDH). Primer sequences as well as the template used for their design can be found in Table 1. PCR products were visualized using ethidium bromide gel electrophoresis using the ChemiDoc system (Bio-Rad, Hercules, CA). Bands were then quantified using Image J software after normalization to GAPDH controls.[10]

### M1 mAChR agonist experiments

The M1 mAChR agonist McN-A-343 (Sigma Aldrich, St. Louis, MO) has been used previously in the study of the muscarinic signaling system in mice.[11,12] Mini osmotic

**Table 1.** Primers used for RT-PCR.

| Target | Forward primer | Reverse primer | Product size | Reference sequence |
|---|---|---|---|---|
| M1 mAChR | 5′-agagaaagaccttctcactgg-3′ | 5′-tgacagtgctgttgacgtagc-3′ | 190 | NM_001112697 |
| M2 mAChR | 5′-gctattaccagtccttacaagaca-3′ | 5′-aagaactgaatatagcactcc-3′ | 500 | NM_203491 |
| M3 mAChR | 5′-tctccattgactatgtggcc-3′ | 5′-acagcgaccgtacttcctcc-3′ | 500 | NM_033269 |
| M4 mAChR | 5′-ggagaagaaggccaagactctgg-3′ | 5′-ataccagctggagtggcagg-3′ | 400 | NM_007699 |
| M5 mAChR | 5′-gtctccgtcatgaccatactcta-3′ | 5′-cagccttttcccagtcagcac-3′ | 300 | NM_205783 |
| LGR5 | 5′-gtctcctacatcgcctctgc-3′ | 5′-ttccaaaggcgtagtctgct-3′ | 880 | NM_010195 |
| BIII tubulin | 5′-catcagcgtatactacaatgaggc-3′ | 5′-agctgctgttcttactctggatg-3′ | 370 | NM_023279 |
| GAPDH | 5′-aactttggcattgtggaagg-3′ | 5′-acacattgggggtaggaaca-3′ | 220 | NM_001289726 |

pumps (Durect Corp., Cupertino, CA) were used to deliver 5 mg/kg/day of McN-A-343, or an equivalent volume of saline in experimental controls, for seven days prior to sacrifice and analysis. Each group consisted of three animals.

Intestinal segments from the distal ileum of each mouse were harvested as above and paraffin sections created. Slides used for morphometric measurements (villus height and crypt depth) were H&E stained, while slides used to measure proliferation were stained for the proliferative marker Ki67 and detected by immunofluorescence after DAPI counterstain. At least 20 villi and crypts were measured per group. Villi were chosen for measurement when intact from crypt-villus junction to crypt-villus junction and central lacteal present. Crypts were measured when intact from crypt-villus junction to crypt-villus junction and at least partial visualization of adjacent villi present. A crypt proliferation index (CPI) was calculated by counting Ki67-positive cells in the crypt and dividing by total cells in the crypt determined by DAPI staining. Fluorescent images were obtained on an inverted confocal microscope (Zeiss, Oberkochen, Germany) and analyzed using ImageJ Software (NIH, Bethesda, MD).

## Results

In whole brain samples, all mAChR subtypes, M1–M5, were successfully amplified as expected (data not shown). These data served to confirm the design of our primers and choice of experimental conditions in a tissue known to express all mAChR subtypes. We next examined whole intestinal segments spanning the small bowel, from proximal jejunum to distal ileum, as regional controls corresponding to each LCM sample. For the segments examined, M1–M4 receptors were found in all regions, while the M5 receptor was only present in the proximal jejunum (Figure 1). We next analyzed LCM samples consisting of crypt-base cells from corresponding regions spanning the small bowel. For LCM samples, the M1 mAChR was found in all regions examined, while M2–M5 mAChRs were absent in all regions (Figure 2). The ability to capture the stem cell regions of the crypt while minimizing the presence of neural elements in LCM samples was determined by PCR using primers for LGR5, BIII tubulin, and GAPDH. Presence of LGR5 signified the presence of intestinal stem cells, while presence of BIII tubulin signified the presence of neural tissue, and finally GAPDH was used as an internal control. Analysis of LCM samples from all regions of the bowel revealed expression of LGR5 and GAPDH mRNA, while BIII tubulin expression was limited to the proximal jejunal and proximal ileal LCM samples (Figure 3).

For *in vivo* experiments, treatment with the M1 mAChR agonist McN-A-343 resulted in statistically significant increases in both morphometric and proliferative parameters in the small intestinal mucosa. When compared to WT controls, increases of approximately 25%, 75% and 25% were observed for villus height, crypt depth, and CPI, respectively (Figure 4).

## Discussion

The expression of all subtypes of muscarinic acetylcholine receptor (mAChRs) mRNA in the crypts of the murine small intestine was investigated with the goal of understanding the mechanisms of muscarinic signaling in the crypt base stem cell compartment. While the ENS and specifically the muscarinic ACh signaling system have both been implicated in intestinal mucosal homeostasis and certain disease states,[4–6,13] the precise mechanisms have not been described. Indeed, previous work from our lab identified a role for the ACh signaling system using mice specifically deficient in one of the five mAChR subtypes, but further experiments were required to clarify the roles of these subtypes in mucosal homeostasis.[14] Given the location of the stem cell niche in the crypt base,[1] and the known projections of cholinergic nerves to the intestinal mucosa,[15,16] we hypothesized that the intestinal crypt base cells would express only specific mAChRs that drive

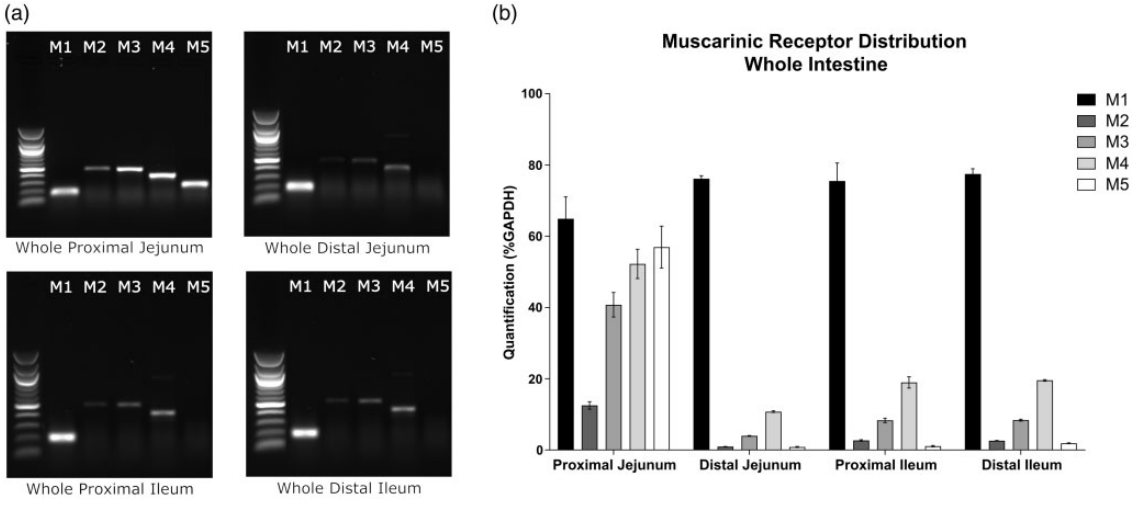


**Figure 1.** Expression of M1–M5 mAChR mRNA determined by RT-PCR. (a) Using whole intestinal samples from each region throughout the bowel as local positive controls, M1–M4 mAChRs were present throughout, while M5 mAChR was limited to the proximal jejunum. (b) Quantification of bands from experiments performed in triplicate, data expressed as mean ± SEM.

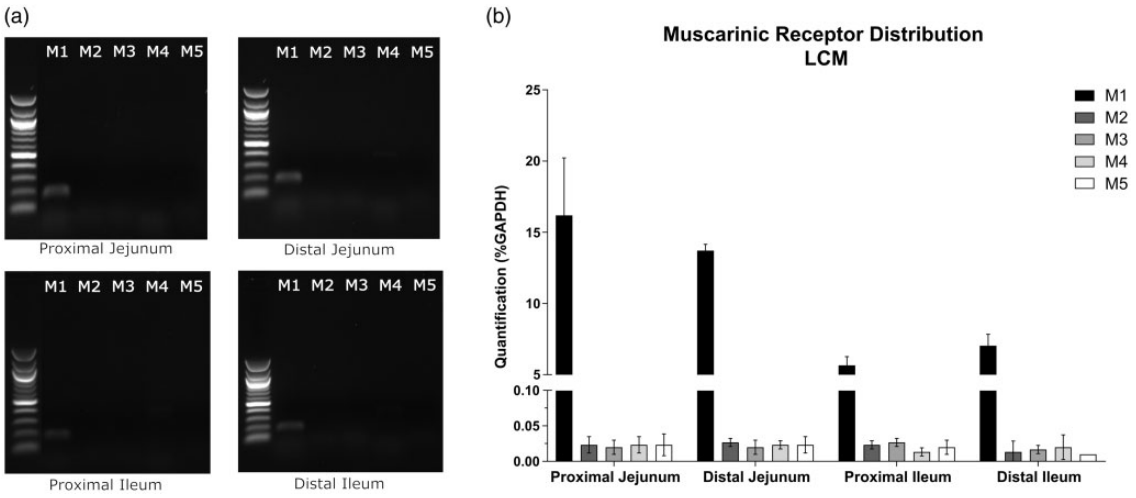


**Figure 2.** Expression of M1–M5 mAChR mRNA from LCM samples determined by RT-PCR. (a) LCM samples spanning the small bowel showed the unique presence of the M1 mAChR in intestinal crypts. (b) Quantification of experiments performed in triplicate reveal expression of the M1 mAChR was at least 100-fold greater than the remaining mAChR's in each region. Data expressed as mean ± SEM.

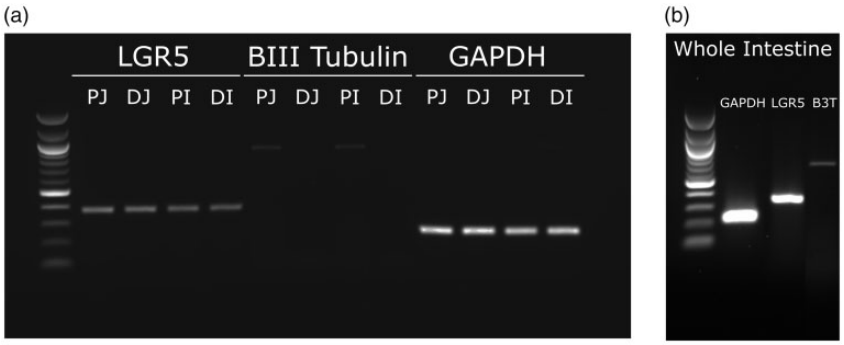


**Figure 3.** An assessment of the quality of LCM samples by determining the presence of markers for intestinal stem cells and neural tissue. (a) All LCM samples expressed LGR5 mRNA, a stem cell marker, confirming the presence of these cells in each sample. BIII tubulin, a neural marker, was present in proximal ileal and jejunal samples but absent from distal ileal and jejunal samples. This suggests the presence of surrounding tissue captured along with crypt cells in the proximal ileal and jejunal samples. GAPDH, a housekeeping gene, is displayed for each sample for reference. (b) Whole intestinal tissue was used as a positive control for GAPDH, LGR5 and BIII tubulin (B3T). PJ: proximal jejunum; DJ: distal jejunum; PI: proximal ileum; DI: distal ileum.

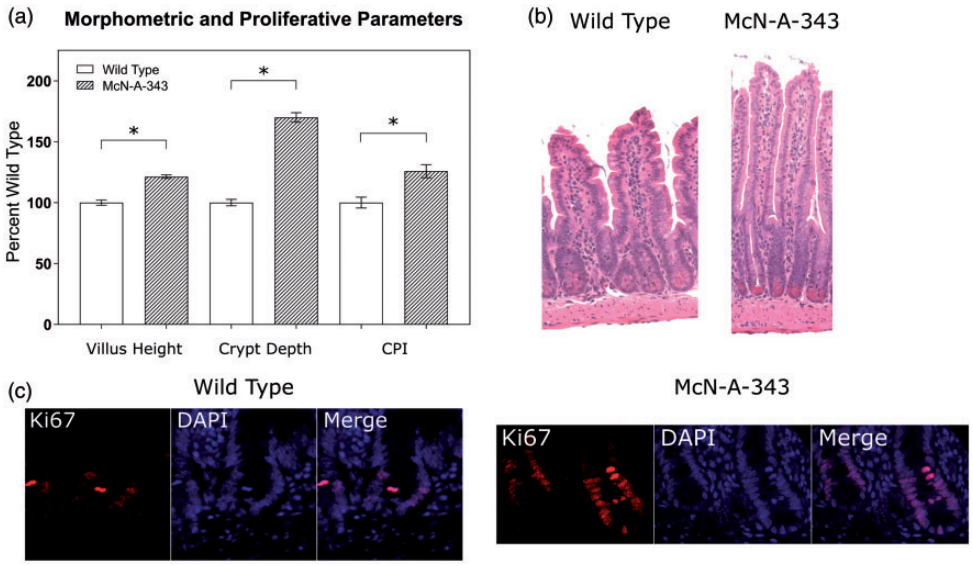


**Figure 4.** Stimulation of the mAChR M1 subtype with McN-A-343 results in significantly increased mucosal growth. (a) The morphometric parameters villus height and crypt depth as well as crypt proliferation index (CPI) are significantly increased when compared to WT controls. Representative images of (b) H&E-stained sections and (c) Ki67 immunofluorescence staining used to calculate CPI demonstrating increased proliferation in the McN-A-343 samples.

proliferation. We used laser capture microdissection (LCM) to specifically isolate cells from this region and RT-PCR to determine mRNA expression of the five subtypes of mAChRs, comparing these samples to whole brain tissue and whole intestinal tissue as non-specific and regional controls, respectively. To determine the quality of our LCM samples, we used markers for intestinal stem cells (LGR5), neuronal tissue (BIII tubulin), and a housekeeping gene (GAPDH). The ideal LCM sample would capture only crypt base stem cells and avoid any surrounding tissue. Thus, a quality LCM sample for this study would express LGR5, but lack BIII tubulin expression, indicating only the crypt base stem cells were in our sample.

In whole intestinal samples, we found M1–M4 mAChRs expressed in all regions of the bowel. M5 mAChR, however, was only found expressed in the proximal jejunum. In LCM samples, we found the expression of M1 mRNA in all regions of the bowel, but the absence of all other receptor subtypes. The expression of M1 mAChR was notably increased in whole intestinal samples analyzed, which could contribute to the findings in our LCM samples, as the starting sample material is considerably smaller. However, our quantification data suggest that these expression differences do not account for our findings. In whole intestinal samples, M1 expression was increased by approximately 10-fold, while in LCM samples, the M1 expression was increased by at least 100-fold.

Our findings suggest the M1 mAChR is critical in ACh signaling in the region of the stem cell niche. Interestingly, previous evidence exists for expression of M2, M3, and M5 mAChRs in intestinal crypts using immunofluorescence,[8,9] but no published data exist localizing M1 to the intestinal crypts. Interestingly, a recent report localizes the M3 receptor to intestinal crypts using immunofluorescence.[17] These conflicting data may be due to the uncertain specificity of muscarinic receptor subtype antibodies for their intended targets.[18,19] Our current data represent novel evidence for the M1 mAChR as the receptor involved in cholinergic signaling within intestinal stem cells. Furthermore, given the potential for cross-reactivity of antibodies used for immunostaining, our data may represent stronger evidence for the presence of M1 mAChR in the murine intestinal stem cell niche.

We determined the mRNA expression of LGR5, an intestinal stem cell marker, BIII tubulin, a marker of neuronal tissue, and GAPDH, a housekeeping gene, in our LCM samples as a means to judge quality. The quality of the distal ileal and distal jejunal samples was found to be optimal, with expression of the M1 mAChR and LGR5 mRNA and absence of BIII tubulin mRNA. This suggests the presence of intestinal crypt cells without the presence of surrounding tissue, specifically, neural tissue that may contain mRNA for muscarinic receptors. For the proximal ileal and proximal jejunal samples, however, LGR5 was present but BIII tubulin was also faintly amplified. It is possible that these samples may have captured surrounding neural tissue, along with intestinal crypt stem cells. Thus, while RT-PCR analysis for all samples consistently demonstrated the presence of M1 mAChR, the presence of BIII tubulin mRNA in the proximal ileal and jejunal samples could arguably be from neural tissue. While this is a possibility and limitation to interpretation of our data, we feel that it is unlikely to account for our results as they remained consistent throughout all samples, whether a small amount of neural tissue was present, as in proximal ileal and jejunal samples, or absent, as in distal ileal and jejunal samples. Furthermore, enteric neurons in the gastrointestinal tract are known to express all mAChR subtypes with the exception of the M5 subtype, whose presence in the GI tract has been variable.[8,20–23] Thus, inclusion of neural tissue in the proximal ileal and jejunal samples resulting in the presence of solely M1 mAChR mRNA would be very unlikely. The data for distal ileal and jejunal samples can be interpreted more clearly, as the presence of the M1 mAChR cannot be attributed to neural tissue, and appears to be present within, or in close proximity to, LGR5-expressing stem cells.

Using these data, we focused our attention on determining the role of the M1 mAChR signaling in intestinal homeostasis *in vivo*. We delivered the M1 mAChR-specific agonist McN-A-343 to WT mice and measured changes to intestinal morphometrics and markers of proliferation. We determined that stimulation of M1 mAChR results in significantly taller villi, deeper crypts, and increased crypt cell proliferation.

These data further clarify the complex signaling mechanisms present in the intestinal epithelium, by localizing a single subtype of mAChR to the area of the intestinal stem cell niche, which when stimulated results in mucosal growth. Our lab has been interested in characterizing these mechanisms since our findings implicating muscarinic cholinergic signaling in serotonin-mediated mucosal growth.[7] The muscarinic cholinergic system is of particular interest as serotonergic neurons are not believed to project to the intestinal epithelium, while cholinergic neuronal projections to the epithelium are well-documented.[15] The unique presence of the M1 mAChR in the stem cell niche suggests a possible common final signaling pathway mediated by cholinergic neurons in the ENS, responding to neural inputs from the ENS.

In conclusion, M1 mAChR mRNA is the sole mAChR mRNA expressed in the intestinal stem cell region of the murine small intestinal crypt, and stimulation of this receptor *in vivo* results in increased mucosal growth. This suggests a critical role of the M1 mAChR in signaling pathways involving intestinal stem cells and represents a promising target of study.

**Authors' contributions:** Study concept and design: CJG, RAC; Acquisition of data: CJG, SJA, VRK; Data Analysis: CJG, SJA, RAC; Drafting and critical revision of manuscript: CJG, RAC.

#### DECLARATION OF CONFLICTING INTERESTS

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