

Distribution of 5-HT₃, 5-HT₄, and 5-HT₇ Receptors Along the Human Colon

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Background/Aims

Several disorders of the gastrointestinal tract are associated with abnormal serotonin (5-HT) signaling or metabolism where the 5-HT₃ and 5-HT₄ receptors are clinically relevant. The aim was to examine the distribution of 5-HT₃, 5-HT₄, and 5-HT₇ receptors in the normal human colon and how this is associated with receptor interacting chaperone 3, G protein coupled receptor kinases, and protein LIN-7 homologs to extend previous observations limited to the sigmoid colon or the upper intestine.

Methods

Samples from ascending, transverse, descending, and sigmoid human colon were dissected into 3 separate layers (mucosa, longitudinal, and circular muscles) and ileum samples were dissected into mucosa and muscle layers (n = 20). Complementary DNA was synthesized by reverse transcription from extracted RNA and expression was determined by quantitative or end point polymerase chain reaction.

Results

The 5-HT₃ receptor subunits were found in all tissues throughout the colon and ileum. The A subunit was detected in all samples and the C subunit was expressed at similar levels while the B subunit was expressed at lower levels and less frequently. The 5-HT₃ receptor E subunit was mainly found in the mucosa layers. All splice variants of the 5-HT₄ and 5-HT₇ receptors were expressed throughout the colon although the 5-HT₄ receptor d, g, and i variants were expressed less often.

Conclusions

The major differences in 5-HT receptor distribution within the human colon are in relation to the mucosa and muscular tissue layers where the 5-HT₃ receptor E subunit is predominantly found in the mucosal layer which may be of therapeutic relevance.

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Key Words

Colon; G protein coupled receptor kinases; Ileum; Receptors, serotonin

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Introduction

Most of the body serotonin stores are found in the gastrointestinal tract, where abnormalities in serotonin release, transport and metabolism are associated with dyspepsia, nausea and vomiting, coeliac disease, inflammatory bowel disease, and irritable bowel syndrome (IBS).¹⁻⁵ Inflammation contributes to these disorders due to underlying inflammatory states in coeliac disease and inflammatory bowel disease and in part because it modulates serotonin levels.^{1,4,6}

The serotonin (5-HT) 4 receptor is expressed in several different cell types in the human colon⁷⁻¹⁶ where it stimulates intestinal activity (ie, prokinetic action) and is a target for treating constipation predominant IBS (IBS-C) and chronic constipation. The 5-HT₄ receptor partial agonist, tegaserod was used for treatment of IBS-C until withdrawn in 2007 as it was associated with rare adverse cardiovascular effects.¹ Currently, the high affinity 5-HT₄ receptor agonist, prucalopride is used to treat chronic or idiopathic constipation refractory to at least two other classes of laxatives in the European Union, Canada, and Australia.¹⁷ Intestinal 5-HT₇ receptors are often located near 5-HT₄ receptors where they augment 5-HT induced responses.^{11,18}

5-HT₃ receptor antagonists decrease colonic motility, secretion and nociception and are used to treat diarrhea predominant IBS (IBS-D).^{1,3} The 5-HT₃ receptor is a ligand gated ion channel composed of 5 subunits that form homomeric (all 3A subunits) or heteromeric (mixture of 3A and 3B, 3C, 3D, or 3E subunits) functionally active channels.¹⁹⁻²¹ All of the human 5-HT₃ receptor subunit genes are expressed in the gastrointestinal tract.²²⁻²⁶ Immunohistochemistry studies have pinpointed co-expression of A, B, C, D, and E subunits in enterocytes and also the nerves of the myenteric plexus^{24,27} indicating that heteromeric 5-HT₃ receptors are likely to form in the human colon.

Although 5-HT₄ and 5-HT₇ receptor splice variants have been identified in separate studies in the small intestine and colon,^{22,28-30} no study has looked at their tissue distribution along the colon. However, one earlier study showed that the 5-HT₃ receptor subunit transcript distribution differed in the sigmoid colon²² providing evidence that specific subunits may be potential targets. Therefore the aim of this study was to investigate the distribution of transcripts of 5-HT receptors and their associated proteins in the ileum and along the length of the colon. Membrane bound receptors are subject to strict processing to be positioned correctly and to regulate their responsiveness following activation. Homo-

logous desensitization involving the G protein coupled receptor (GPCR) kinases (GRKs) down regulates surface expression of 5-HT₄ receptors.³¹⁻³³ Proteins such as protein LIN-7 (LIN7) homolog A to C of *Caenorhabditis elegans* (also known as Veli1-3 in mammals) are likely to be involved in processing 5-HT₄ and 5-HT₇ receptors to the plasma membrane.³⁴⁻³⁷ Receptor interacting chaperone 3 (RIC3) assists cell surface assembly of 5-HT₃ receptors.^{38,39} Therefore, transcripts of RIC3, LIN7 homologs, and GRKs were measured as these proteins contribute to receptor processing. Although subtle differences in distribution of the receptors and associated proteins occurred along the colon, only the 5-HT₃ receptor subunits exhibited any major differences in expression between the tissue layers which may be of therapeutic relevance.

Materials and Methods

Human Tissue

Full thickness specimens from different regions of the intestine of 24 patients (11 male and 13 female ranging in age from 50 to 88 years [median 73]) were collected immediately following surgical resection and transported to the laboratory in 4°C Krebs-Henseleit solution. The patients were undergoing surgical resection for colonic cancer and the pathologist indicated that the specimens obtained as far from the tumor as possible appeared to be disease free following gross visual examination. The mucosa and associated mesentery plus fat were removed before the longitudinal muscle bands (taeniae coli) were dissected and the remaining intertaenial tissue was cut into strips in the orientation of the circular smooth muscle. All tissue was stored in RNAlater[®] (Ambion, Austin Texas, USA) at -80°C. The project was approved by the Human Research Ethics Committee of the hospital and academic institute and all patients gave informed consent prior to surgery.

RNA Extraction

Approximately 18-25 mg excised tissue was homogenized using a PRO 200 homogenizer (PRO Scientific Inc, Oxford, CT, USA) and total RNA was extracted using RNeasy Fibrous kit (Qiagen, Chadstone Center, Victoria, Australia) and Turbo DNA-free[™] Kit (Life Technologies, Mulgrave, Victoria, Australia) was used to degrade any contaminating DNA. RNA was quantified pre and post-DNase treatment with a Nanodrop 1000 using ND-1000 software (Thermo Scientific, Wilmington,

DE, USA). The reverse transcription step was carried out using 1 µg of DNase-treated RNA, SuperScript[®]III Reverse Transcriptase (Life Technologies) and oligo dT₁₅ primers in a total reaction volume of 20 µL. Separate negative reverse-transcriptase controls were included for every reaction.

Polymerase Chain Reaction Conditions

In end-point polymerase chain reactions (PCR), the primers described in a prior study²² were used to amplify GRK, 5-HT₄, and 5-HT₇ receptor genes while the following primers were used to amplify LIN7A-C and glyceraldehyde dehydrogenase (GAPDH) genes. GAPDH (NM_002046.5) forward 5'-ACCACAGTC-CATGCCATCAC-3' (714-734) and reverse 5'-TCCACCAC-CCTGTTGCTGTA-3' (1165-1146); LIN7A (NM_004664.2) forward 5'-CAGCTAGTGAAGGCCACTCC-3' (486-505) and reverse 5'-GCAGCTGGTCTCCTCTTTTG-3' (659-639); LIN7B (NM_022165.2) forward 5'-CAGCTTTATGACACGCTGGA-3' (210-229) and reverse 5'-GATGACCCGG-GAGATGT-3' (413-397); and LIN7C (NM_018362.3) forward 5'-AACAGAAGAGGGCCTTGGAT-3' (516-496) and reverse 5'-GCGGCTTTCAGCAGTTCTAC-3' (321-340) which will produce products of 451 (for all 4 splice variants), 174, 203, and 195 bp respectively. It should be noted that the LIN7A primer set is within one exon. Amplifications were undertaken in a MyCycler thermal cycler (Bio-Rad Laboratories, Inc, Hercules, CA, USA) and a hot start involving 15 minutes at 94°C was made. To keep within an exponential range during amplification, 20 to 25 cycles were made with denaturation at 94°C, annealing at 55°C, and extension at 72°C all for 1 minute. Products were visualized following separation on agarose gels and staining with GelRed[™] nucleic acid stain (Biotium, Hayward, CA, USA).

Quantitative PCR was undertaken using similar amplification conditions as described in a prior study²² except that the 30 µL reactions contained 1 µL cDNA, 0.5 µmol/L forward and reverse primers, 4 mmol/L MgCl₂, and 2× SensiMix SYBR Green PCR Master Mix (Bioline, Sydney, NSW, Australia). The cDNA was amplified by 1 cycle at 95°C for 15 minutes followed by 36 cycles of 95°C for 15 seconds (denaturing), 55°C for 20 seconds (annealing), and 72°C for 25 seconds (extension) using a C1000[™] Thermal Cycler and CFX96[™] Real-Time System (Bio-Rad Laboratories). Melting point analysis indicated that only a single product was produced in each reaction and confirmed by preliminary runs with gel electrophoresis which also established that only one product of the predicted size was produced. Control runs of all PCR experiments contained non-tem-

plate controls and negative reverse transcriptase controls consisting of RNA samples where reverse transcriptase was not added (such that no cDNA was produced) to test for DNA contamination.

Statistical Methods

Each quantitative PCR sample was analyzed in duplicate and efficiency of reactions was determined using linear regression of the Log (fluorescence) per cycle number data with the LinRegPCR program⁴⁰ and ranged from 2.008 ± 0.006 (β-actin) to 1.877 ± 0.008 (5-HT₃ receptor C subunit [HTR3C]). Expression data was calculated relative to β-actin and GAPDH and expressed as the following ratio: Ratio = (Efficiency_{reference})^{Ct_{reference}} / (Efficiency_{sample})^{Ct_{sample}}, where Ct is the crossing point threshold of the sample for the amplified genes.

Relative expression data was further analyzed after log transformation using one-way ANOVA followed by Tukey's multiple comparisons test. One-way ANOVA and Tukey's multiple comparisons were used to analyze the number of patients expressing genes detected using end point PCR. The number of observations used to derive mean values is expressed by n and arithmetic mean values are given as mean ± SEM.

Results

Distribution of 5-HT₃ Receptor Subunits in the Human Intestine

The distribution of 5-HT₃ receptor subunits was examined using quantitative reverse transcript PCR on samples obtained from throughout the length of the colon and the ileum area of the small intestine. In all regions, expression was examined in both the mucosal and muscular tissue layers and reported relative to expression of β-actin. GAPDH was used as a second house-keeping gene for comparison with a previous study where the relative expression of the 5-HT₃ receptors in the sigmoid colon was reported.²² Control RNA samples incubated without reverse transcriptase and then amplified with GAPDH primers were also undertaken to demonstrate that there was no DNA contamination of the RNA extractions (data not shown). GAPDH levels of expression were consistently and significantly higher than those of the 5-HT₃ receptor subunits or RIC3 in both ileum and colon (*P* < 0.05 one-way ANOVA; Fig. 1) which is consistent with the previous study.²² The 5-HT₃ receptor D subunit

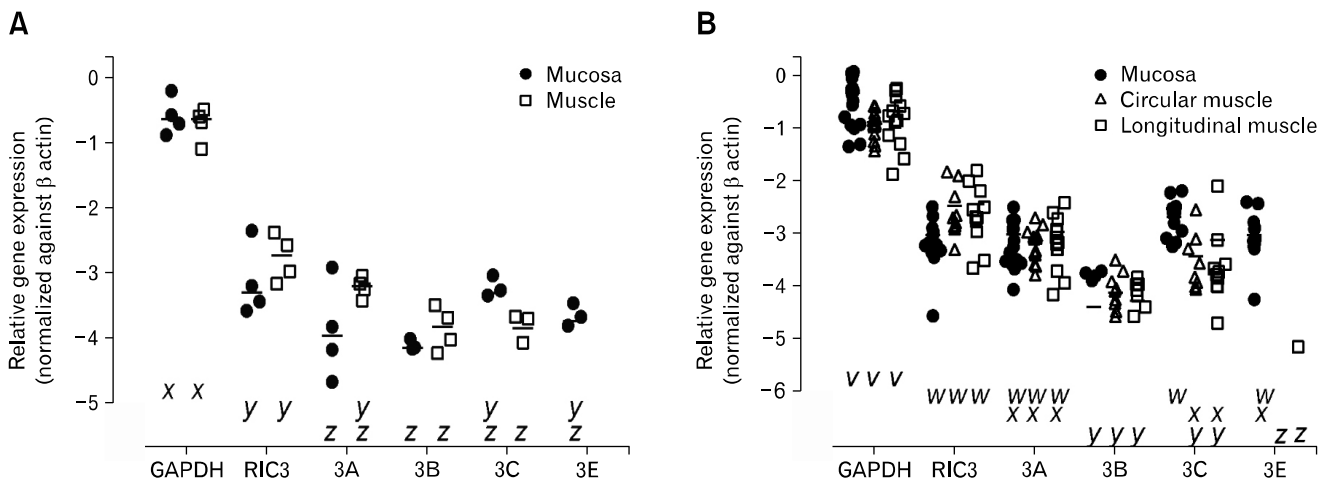


Figure 1. Distribution of serotonin type 3 (5-HT₃) receptor subunits and receptor interacting chaperone 3 (RIC3) transcripts in human intestinal tissue layers. (A) Comparison of the relative expression levels of transcripts of RIC3 and 5-HT₃ receptor subunits in the mucosal and muscle tissue layers in the human ileum (n = 4). (B) Comparison of the relative expression levels of transcripts of RIC3 and 5-HT₃ receptor subunits in the mucosal and muscle layers (circular and longitudinal) in tissue samples obtained from throughout the human colon (n = 16). Data are expressed as a ratio relative to β-actin as described in the Material and Methods section. Bars indicate the mean. 5-HT₃ receptor D subunit (HTR3D) transcripts were not detected in any tissue tested. RIC3 and 5-HT₃ receptor subunit transcripts are expressed at significantly lower levels than glyceraldehyde dehydrogenase (GAPDH) transcripts (*P* < 0.001 one-way ANOVA followed by Tukey’s multiple comparisons test) in all tissues. The letters above the x-axis (v, w, x, y, and z representing the highest to lowest level respectively) indicate that the transcripts are found at significantly different levels (*P* < 0.05 one-way ANOVA followed by Tukey’s multiple comparisons test) in the ileum or colon tissue layers (ie, transcripts in the different layers with an x underneath are expressed at the same level; x is the highest and z the lowest level in [A] while v is the highest and z the lowest level in [B]).

was consistently not detected in either ileum or colon samples which is in agreement with prior studies where transcripts of HTR3D were only evident at very low levels.^{23,25} In the ileum, RIC3 and 5-HT₃ receptor A subunit transcripts were found in all samples with the other subunits being less prevalent (Fig. 1A). Transcript levels of RIC3 were significantly higher than 5-HT₃ receptor B subunits in both mucosa and muscle layers and also the mucosa levels of the A subunit and the C subunit in the muscle layer. No significant differences were observed in the expression levels of the 5-HT₃ receptor subunits with the major exception that the E subunit was only found in the mucosa layer (Fig. 1A).

The colonic tissue was dissected into mucosa, circular and longitudinal muscular layers and a similar distribution of the specific transcripts occurred in the different areas (Supplementary Fig. 1). Therefore, the regional data was pooled to see if there were any differences in gene expression at the tissue layer level (Fig. 1B). Generally a similar distribution pattern of RIC3 and the 5-HT₃ receptor subunits in the colonic tissue layers was observed to that seen in the ileum. However some variations were seen in 5-HT₃ receptor subunit expression between the different tissue layers (Fig. 1B). The 5-HT₃ receptor B subunit was ex-

pressed at significantly lower levels than RIC3 or the A subunits in all tissue layers. The mucosal levels of the C subunit were significantly higher than the B subunit in any tissue and the C subunit in the muscular layers. The 5-HT₃ receptor E subunit transcripts were nearly always only detected in the mucosa samples at levels significantly greater than the B subunit (Fig. 1B). Only one longitudinal muscle sample showed any E subunit expression (Fig. 1B), so to determine if the 5-HT₃ receptor E subunit was expressed in the muscle layers but below the reliable detection levels of the quantitative PCR conditions, the amplified PCR samples were run in gels (Supplementary Fig. 2). Only very low levels of expression were observed in two additional colon mucosa samples and two longitudinal muscle samples as well as an additional ileum muscle sample.

Distribution of 5-HT₄ and 5-HT₇ Receptors in the Human Intestine

Adjacent tissue samples to those used in the analysis of the 5-HT₃ receptor and RIC3 gene expression were used to examine the distribution of 5-HT₄ and 5-HT₇ receptors. Since several splice variants with overlapping 3’ coding regions exist for the 5-HT₄ and 5-HT₇ receptors, their distribution was examined us-

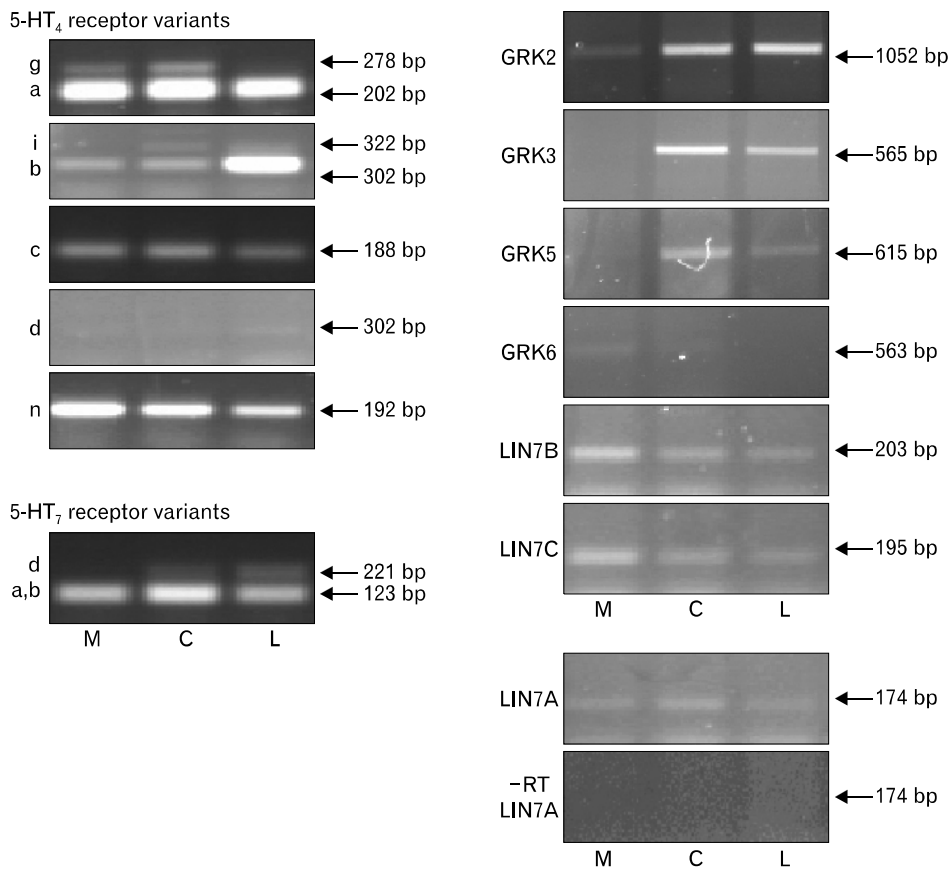


Figure 2. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of expression of protein LIN-7 homologs G protein coupled receptor kinase (GRK), serotonin type 4 (5-HT₄) and 5-HT₇ receptor gene products in the human descending colon of one patient. The products of predicted sizes are indicated by arrows and the size was correlated to 100 bp (molecular weight) markers run on 1.5% agarose gels stained with GelRed. A negative control is shown for the LIN7A sample as the primers are within one exon. Letters represent samples obtained from M (mucosa), C (circular muscle), and L (longitudinal muscle).

ing partial or nested PCR. GAPDH expression was used as a control for this analysis as it has previously been shown to be comparable between human colonic tissue layers¹¹ (Fig. 1 and Supplementary Fig. 3). An example of the expression of different transcripts following end point RT-PCR analysis is shown for a descending colon sample (Fig. 2). The samples were obtained from ileum, ascending, transverse, descending and sigmoid colon and overall the distribution of transcripts appeared independent of the region of the colon and similar across the types of tissue layers (Supplementary Table 1). Therefore the number of patients expressing any of the genes was compared with the colon tissue layers (Fig. 3). One-way ANOVA indicates that the only differences are in the expression of the gene transcripts ($P < 0.05$) and not the tissue layer. For instance, 5-HT₄ receptor d and g splice variants are significantly less likely to be detected in mucosa and longitudinal muscle compared to the 4a, b, c, and n splice variants ($P < 0.05$ one-way ANOVA; Fig. 3). Like the 5-HT₄ a, b, c, and n splice variants, the 5-HT₇ receptor splice variants were widely expressed in all regions and tissues studied. Some minor differences are evident with respect to GRK distribution. For example, GRK6 is less likely to be expressed in the ileum or ascend-

ing and transverse colon compared to the descending and sigmoid colon (Supplementary Table). Both GRK5 and GRK6 are expressed less often in all of the tissue layers compared to the 5-HT₇ receptor a/b variants and in the mucosa and longitudinal muscle than the 5-HT₄ receptor a, b, c and n variants ($P < 0.05$, one-way ANOVA; Fig. 3). However, no differences in the frequency of expression were observed between the different GRK or LIN7 genes in the different tissue layers ($P > 0.05$, one-way ANOVA; Fig. 3).

Discussion

Despite many similarities in its general functionality, the human intestinal tract exhibits considerable differences to small animal models such as mice, rats, and guinea pigs.⁴¹ Serotonin receptors for instance are widespread throughout the gastrointestinal tract in small animal models and humans but the proportion and type of receptors at particular regions are different.⁴¹⁻⁴³ In addition, human 5-HT receptors are considerably different in their composition as evident by the diverse splice variants in 5-HT₄ and 5-HT₇ receptors and the additional 5-HT₃ receptor

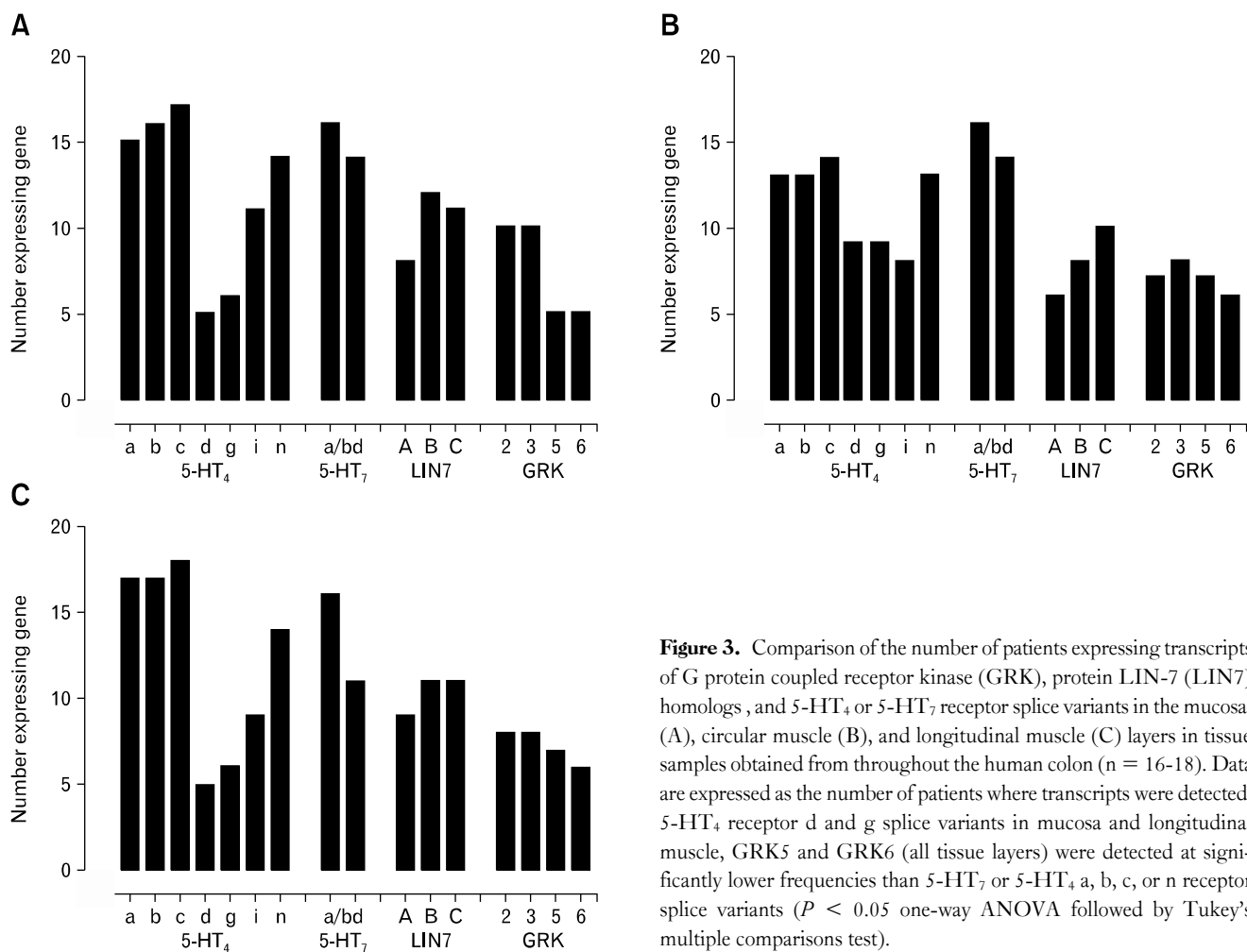


Figure 3. Comparison of the number of patients expressing transcripts of G protein coupled receptor kinase (GRK), protein LIN-7 (LIN7) homologs, and 5-HT₄ or 5-HT₇ receptor splice variants in the mucosal (A), circular muscle (B), and longitudinal muscle (C) layers in tissue samples obtained from throughout the human colon (n = 16-18). Data are expressed as the number of patients where transcripts were detected. 5-HT₄ receptor d and g splice variants in mucosa and longitudinal muscle, GRK5 and GRK6 (all tissue layers) were detected at significantly lower frequencies than 5-HT₇ or 5-HT₄ a, b, c, or n receptor splice variants (*P* < 0.05 one-way ANOVA followed by Tukey's multiple comparisons test).

subunits.^{1,19,44} The function and pharmacological responses of 5-HT receptors in the intestine also varies between small animal models and humans^{15,41,45,46} and therefore this study was undertaken to gain an insight to the distribution of 5-HT₃, 5-HT₄, and 5-HT₇ receptors expressed in the human ileum and throughout the human colon. Although subtle differences in the regional distribution of the 5-HT receptors occurred, the main differences were between the mucosal and muscular tissue layers.

Quantitative and end point PCR were used to assess the distribution of 5-HT₃, 5-HT₄, and 5-HT₇ receptors using samples obtained from surgical resection that appeared to be disease free following gross visual examination. Tissue samples were stored in RNeasy[®] at -80°C until use. Only samples that contained readily detectable levels of GAPDH (and β-actin for quantitative PCR) and were not contaminated by genomic DNA (no bands detected in the negative reverse transcript assays) were processed further to exclude the probability of undetectable expression due

to storage-related RNA degradation (Supplementary Fig. 3). The robustness of the quantitative PCR was ensured by adhering to the MIQE guidelines⁴⁷ and samples were run on gels to ensure that only the expected products were generated (Supplementary Fig. 2). The end point PCR studies were designed to ensure that the products were still within the linear amplification range. Where expression levels were low (eg, 5-HT₄ receptor splice variant d; Fig. 2), independent observers confirmed the presence of detectable bands in the gels.

The distribution of 5-HT₃ receptor A subunits is in keeping with previous studies where this subunit was widespread and thought to reflect the distribution of functional 5-HT₃ receptor subunits.^{11,22,23,25,48} The HTR3B gene which encodes for the canonical 5-HT₃ receptor B subunit was expressed at lower levels than HTR3A in all regions of colon but at similar levels in the ileum (Fig. 1). The actual occurrence of HTR3B in human colon and ileum (Supplementary Fig. 1) was almost 50% less than

the total occurrences of HTR3A and agrees with previous findings where lower levels but no distinct patterns of expression were observed.^{23,25,27} Interestingly, the expression and occurrence of the 5-HT₃ receptor C subunit was similar to HTR3A while HTR3E was predominantly restricted to the mucosa confirming prior observations in the colon, small intestine and stomach.^{22,23,25,26,38,49} The mucosa plays a role in fluid transport, while the muscular layers are involved with motility so it is possible that physiological alterations in the different colon tissue layers may result in selective colon disease or dysfunction. Immunoreactants to both 5-HT₃ receptor C and E subunits have been identified as being co-expressed within the human colon (enterocytes, myenteric plexus and muscular layer).²⁴ Together these findings are suggestive that associations of 5-HT₃ receptor A subunits occur with C and/or E subunits in the gastrointestinal tissues. In fact, genetic studies have revealed associations of HTR3C and HTR3E with several clinical conditions.⁵⁰⁻⁵³ Interestingly, a single nucleotide polymorphism in the 5-HT₃ receptor E subunit that is associated with female IBS-D where the 5-HT₃ receptor subunit is up-regulated by microRNA (miR-510) co-expressed with the E subunit in gut epithelium enterocytes.⁵¹

The 5-HT₄ receptor splice variants have different preferences to ligands as shown by different potency and binding affinities for various 5-HT₄ receptor agonists^{44,54-57} and their distribution may influence drug responses. 5-HT₄ receptor transcripts have been identified in human mucosa biopsies obtained from the duodenum, ileum and colon and this mucosal expression contributes to luminal responses in model animal systems.¹⁰ There are no significant patterns in the expression of 5-HT₄ or 5-HT₇ receptors although the number of patients expressing the 5-HT₄ receptor d and g splice variants is consistently lower (Fig. 3). The 5-HT₄ receptor d splice variant in particular has been shown to have low expression levels before in different parts of the body such as the central nervous system.³⁰ It would be of interest to learn if changes in distribution of 5-HT₄ receptor splice variants occur in gastrointestinal disorders as the 5-HT₄ receptor a and b splice variants are down regulated while the d variant is upregulated in adenomas.⁵⁸ The 5-HT₇ receptor splice variants in this study were expressed in all of the different tissue layers. The overall expression patterns of 5-HT₄ and 5-HT₇ receptors are in accordance with their known functional properties in the human colon to regulate ascending contraction and descending relaxation to generate peristaltic reflexes resulting in bowel movement.^{7-16,18} 5-HT₇ receptors are also found on macrophages and have been implicated as a potential target for treating intestinal inflamma-

tory disorders.^{59,60}

Serotonin induces desensitization (tachyphylaxis) at different rates and magnitudes in different tissues and this is the case with 5-HT₄ receptor agonists as highlighted by prucalopride desensitizing pig atrium but not pig stomach 5-HT₄ receptors.⁶¹ Different GRKs are associated with 5-HT₄ receptor desensitization depending on the tissue type in the rat and significantly these GRKs are not necessarily those predicted by studies using recombinant proteins expressed in cell culture.³² GRK2 and GRK3 were relatively well distributed in all tissue layers while GRK5 was less common and GRK6 was absent in the ileum but present in the descending and sigmoid colon, so there may be regional and tissue layer variation in the GRKs involved in 5-HT₄ receptor desensitization in the human colon. The distribution of LIN7 homologs in the human colon is of interest as LIN7C (Veli 3) has been shown to interact with the 5-HT_{4a} receptor splice variant.³⁵ In the human intestine, all 3 LIN7 homologues were present in the different tissue regions except the region of the transverse colon tissue layers. However, detection of the LIN7 homologues may be limited by their occurrence at pre-synaptic (axonal) and postsynaptic (dendritic) subcellular compartments.³⁶

In conclusion, this study demonstrates that the major differences in the localization of 5-HT receptors within the human colon are in relation to the mucosa and muscular tissue rather than the region of the intestine. The fact that the study proteins are distributed along the intestine, only serves to emphasize how dominant the receptors and their associated proteins are, and so any dysfunction of one is likely to manifest seriously in clinical conditions. Only minor differences were observed in the distribution of 5-HT₄ and 5-HT₇ receptors and their splice variants. Differences in the distribution of 5-HT₃ receptor subunits were evident with A, B, and C subunits occurring in all tissues whereas the E subunit was only significantly observed in the mucosal layer. The findings suggest that it may be possible to target 5-HT₃ receptors in the mucosal or muscular layers if subunit specific molecules can be generated to cater for different colon diseases or dysfunction.

Supplementary Materials

Note: To access the supplementary table and figures mentioned in this article, visit the online version of *Journal of Neurogastroenterology and Motility* at <http://www.jnmjournal.org/>, and at <http://dx.doi.org/10.5056/jnm14157>.

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