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Mini-Review

Muscarinic receptor signaling and gastrointestinal tract cancer

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ABSTRACT

Expressed in a wide array of mammalian organs and tissues, acetylcholine receptors are categorized structurally and functionally as either muscarinic or nicotinic. Muscarinic receptors are further sub-categorized into five subtypes, designated M1R–M5R. As members of the guanine nucleotide binding (G) protein-coupled receptor superfamily muscarinic receptors transmit signals by activating G proteins that regulate downstream protein kinase cascades that alter gene expression or otherwise modulate cell function. Coupling to G proteins further distinguishes receptor subtypes as either $G_{q/11}$-bound (M1R, M3R, M5R) or $G_{i/o}$-bound (M2R, M4R). Gastrointestinal epithelial cells express M1R and M3R primarily. Herein, we focus on exploring functional differences between M1R and M3R activation, particularly with respect to responses in normal epithelium and different gastrointestinal cancers. Variable responses may result from allosteric binding sites, bitopic ligands, differential receptor distribution, and sequence variations that modulate the affinity and stability of ligand binding. Notably, major dissimilarities in the progression of gastrointestinal pathology result from M1R versus M3R expression or activation. Autocrine and paracrine M3R activation or M3R overexpression promotes neoplasia in the stomach and colon; blocking M3R expression or activation attenuates cancer progression. In contrast, M1R activation appears to act as a tumor suppressor; knockout of both M1R and M3R expression negate the anti-colon tumor effects of M3R knockout alone. Dynamic interplay between M1R and M3R activation and distinct post-receptor signaling in the gastrointestinal tract offers novel therapeutic possibilities. A more complete understanding of muscarinic receptor subtype function and action will likely result in innovative approaches to treating gastrointestinal disorders, including cancer.

KEYWORDS: M1R, M3R, cancer, gastrointestinal, muscarinic receptors, cell signaling.

1. Introduction

Receptors for acetylcholine comprise two large families; ligand-gated ion channel *nicotinic* receptors are structurally and functionally distinct from G protein-coupled receptor (GPCR) *muscarinic* receptors. Nicotinic receptors are beyond the scope of this review – those interested may consider recent reviews of nicotinic receptor physiology [1-3].

Muscarinic receptors (MR's) are sub-categorized structurally as M1, M2, M3, M4, and M5 receptors [4]. Functionally, odd-numbered M1, M3, and M5 receptors interact with guanine nucleotide binding proteins in the $G_{q/11}$ family, whereas even-numbered M2 and M4 receptors interact with the $G_{i/o}$ family [5]. While we know much about the wide-ranging effects of muscarinic receptor activation in multiple organ systems, we know very little about the disparate effects of M1 and M3 muscarinic receptor activation on the gastrointestinal (GI) tract and liver. Hence, the

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focus of the present review is functional differences between M1R and M3R expression and activation in GI cancers.

2. Receptor structure

As GPCRs, MR's possess seven transmembrane α-helical segments with three extracellular and three intracellular loops between the helices as well as an extracellular N-terminus and an intracellular C-terminus [6, 7]. The MR intracellular C-terminus couples to heterotrimeric G-proteins comprised of α, β, and γ subunits. Receptor activation splits these heterotrimers into Gα- and Gβγ-subunits [8].The precise three-dimensional structure of these receptors was difficult to obtain *via* crystallization due to changes in conformation and receptor instability outside the cell membrane's hydrophobic environment, but they have recently been solved [9]. Only a few GPCRs have been crystallized and studied with x-ray crystallography: rhodopsin, A_{2A} adenosine, and β1 and β2 adrenergic receptors. Much of what is known regarding MR structure was derived from site-directed mutagenesis *via* alkylating agents such as propylbenzilylcholine and acetylcholine mustards [7]. Resulting data reveal the orthosteric binding site of MR's is similar to that of rhodopsin and β adrenergic receptors. Recently, M2R and M3R X-ray crystallographic structures were determined, with M3R crystallized in a complex with tiotropium [5, 9].

Site-directed and random mutagenesis revealed several amino acid residues that play an important role in stabilizing MR's. Substitutions of these residues result in decreased or absent levels of MR expression [7]. In addition to their role in receptor stability, amino acid residues also form orthosteric and allosteric ligand binding sites – both are present on MR's. The primary MR extracellular ligand, acetylcholine, binds to the orthosteric site. It is thought that other ligands that alter receptor function bind at a separate allosteric binding site. A homology model for M1R based on bovine rhodopsin reveals the most important amino acids for interaction with acetylcholine are $Tyr^{3.33}$, $Thr^{5.39}$, $Thr^{5.42}$, $Tyr^{6.51}$, and $Tyr^{7.394}$. Other amino acid residues are important for binding acetylcholine, but they may also interact with antagonists or facilitate introduction of ligands to the orthosteric binding site. Modifications of the amino acid sequence also affect the binding affinity of the orthosteric sites for ligands. While amino acids in the orthosteric binding site are conserved amongst MR subtypes, when comparing sequence alignments with X-ray crystallographic information, residues in a solvent-accessible extracellular vestibule are less conserved between subtypes [9].

MR's have at least two allosteric binding sites. The "prototypical modulator site" is the best described; its ligands interact with all five MR subtypes. However, while these prototypical ligands bind at the allosteric sites of all subtypes, they do so with varying affinities. Ligands that bind to both the orthosteric and allosteric binding sites simultaneously are termed bitopic ligands or dual steric activators [7, 10]. Previous studies showed that MR subtype specificity is achievable through such bitopic ligands [10].

3. Post-muscarinic receptor signaling

Downstream actions of MR activation are mediated primarily by three major types of Gα-subunits: $G_{q/11}$, $G_{i/o}$, and G_s. $G\alpha_q$ proteins activate phospholipase Cβ (PLC), and G_i and G_s regulate adenylyl cyclase [6]. The Gβγ-subunit may modulate ionic conductance through phospholipase Cβ upon activation of M2R and M3R. By interacting with G_i, M2 and M4 receptors inhibit adenylyl cyclase to increase the opening duration of potassium ion and non-selective cation channels. In contrast, M1, M3, and M5 receptors activate G_q to convert phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) *via* PLC, which increases intracellular calcium levels. While odd-numbered MR's all increase intracellular calcium, they do so with different efficiencies; M1R most efficiently activates Gα, followed by M3R then M5R (Figure 1).

In addition to the second messengers IP3 and DAG, RhoA, protein kinase C (PKC), phosphoinositide-3 kinase (PI3K), non-receptor tyrosine kinases (nRTK), serum response factor (SRF), and mitogen-activated protein kinases (MAPK) may mediate effects of GPCR activation. Of note, RhoA and SRF activation appears to uniquely follow M1R but not M3R activation [8].

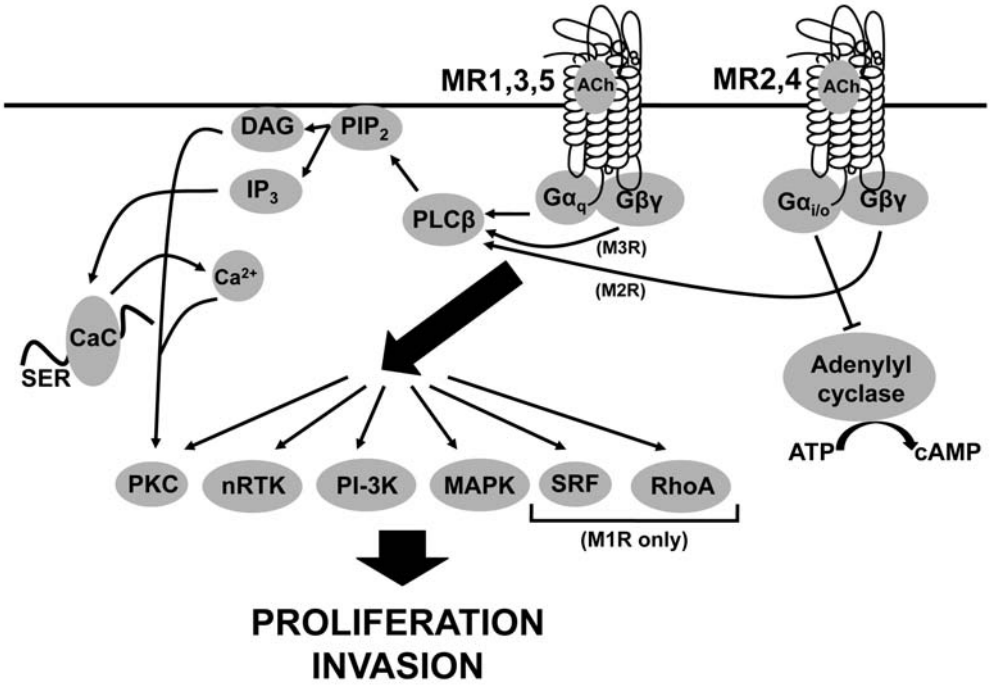

Figure 1. Muscarinic receptor signaling pathways.

MR: muscarinic receptor, DAG: diacylglycerol, PIP_2: phosphatidylinositol 4,5-bisphosphate, IP_3: inositol 1,4,5-trisphosphate, PLCβ: phospholipase Cβ, $G\alpha_q$: G-protein subunit alpha subtype q, Gβγ: G-protein subunit βγ, $G\alpha_{i/o}$: G-protein subunit alpha subtype i/o, SER: smooth endoplasmic reticulum, CaC: calcium channel, Ca^{2+}: ionized calcium, PKC: protein kinase C, nRTK: non-receptor tyrosine kinase, PI-3K: phosphoinositide-3 kinase, MAPK: mitogen-activated protein kinase, SRF: serum response factor, RhoA: Ras homolog gene family, member A, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate.

SRF plays a role throughout the GI tract, including effects on esophageal and gastric ulceration; colon, gastric, pancreatic, and liver cancer progression; liver injury; and pancreatitis [11, 12].

While M3R is the primary mediator of smooth muscle contraction in the GI tract, synergistic interactions between M3R and both M2R and transient receptor potential canonical (TRPC)-encoded proteins potentiate smooth muscle contraction. While both M1R and M3R activate the same G protein pathway to increase intracellular calcium, they may differ in their interactions with other molecules. M1R can interact with TRPC6 activation through complex formation of M1R with PKC. Additionally, in contrast to M3 and M5 receptors, M1R does not activate T-type calcium channels [8]. At pre-synaptic neuronal junctions in the GI tract, M1R activation potentiates neurotransmitter release *via* PKC, while PKA mediates M2R and M4R activation-induced inhibition of neurotransmitter release.

MR conformation may also mediate signaling specificity depending on the ligand bound to the allosteric site. Allosteric ligands do not simply activate or suppress MR's - their unique chemistries change the conformational shape of the receptor and affect downstream signaling [10].

4. Expression

MR's exert various downstream effects on multiple tissue types, based in part on their differential expression (Table 1) [13]. While the bladder expresses all five MR subtypes, primarily M3R activation mediates detrusor muscle contraction, despite the greater abundance of M2R. In salivary glands, M3R predominates in the parotid gland, and is primarily responsible for salivary secretions. However, variable activation of M1R and M3R determines the quantity and

Table 1. Examples of differential expression of muscarinic receptor subtypes [13, 21].

Organ	Muscarinic receptor subtype expressed	Actions of muscarinic receptor activation	Primary mediator
Brain	M1, M2, M3, M4, M5	Memory, cognition	M1, M2
Eye	M1, M2, M3, M4, M5	Iris sphincter	M3, M5
Salivary gland	M3 > M1	Saliva production	M3
Heart	M1, M2, M3, M5	Decrease heart rate, contractility (M2), tachycardia (M1)	M1, M2
GI tract	M1, M2 > M3	Gastric acid secretion, smooth muscle contraction	M1, M3
Bladder	M2 > M3 >> M1, M4, M5	Detrusor muscle contraction	M3

viscosity of saliva. The brain and eye express all five MR subtypes. In the brain, M1R and M2R are primarily involved with memory and cognition. In the eye, M3R is most abundant, controlling contractility in the iris sphincter. M2R activation in the heart causes negative inotropic and chronotropic effects [14]. While M2R outnumber M3R, activation of the latter predominantly mediates M3R smooth muscle contraction in the GI tract [13].

Interacting MR's are expressed differentially within the GI tract. Blood vessels express M1R, M3R, M4R, and M5R; M1R, M3R, and M5R mediate nitric oxide production (relaxing smooth muscles), inflammation and proliferation. GI tract smooth muscles express M2R and M3R, controlled by neurons expressing M1R and M4R. Gastric secretion is mediated by M3R [8] and M5R [15] for acid and M1R and M3R for pepsinogen [8, 16].

5. Functional considerations

In the GI tract, MR's influence many functions including gastric acid, bicarbonate, mucus, and pepsinogen secretion; smooth muscle contraction and relaxation; fluid transport; stem cell activation; inflammation; and Paneth cell activation, which play a role in GI immunity. MR's also mediate inflammation, with M1R stimulating leukocyte chemotaxis and M3R stimulating monocyte differentiation and epithelial cell release of inflammatory molecules [8]. These actions were discovered primarily through agonist-antagonist studies and MR subtype-deficient mice.

5.1. Regulation of secretion in the GI tract

Interactions between enterochromaffin-like (ECL) cells *via* histamine release, G-cells *via* gastrin release, and D-cells *via* somatostatin release regulate acid secretion from gastric parietal cells; all four of these cell types express MR's. MR activation stimulates G-cells and inhibits D cells though it is unclear which MR subtype is activated. ECL cells release histamine in response primarily to gastrin, however a portion also responds to M1R activation. Parietal cells can also respond directly to M3R activation for gastric acid secretion [8]. In mice, Aihara *et al.* demonstrated that M3R on parietal cells and M5R possibly in the submucosal plexus mediate gastric acid secretion, whereas M1R plays no role [15]. In contrast, activation of M1R and M3R in gastric chief cells stimulates pepsinogen release [16]. The evidence for muscarinic control of duodenal bicarbonate secretion is inconclusive; studies report conflicting results. There appears to be neural control of bicarbonate secretion. Bicarbonate-secreting cells express M3R, which may mediate bicarbonate secretion, but experimental results are inconclusive. Vagal stimulation does not alter small intestinal goblet cell mucus secretion, whereas it is increased by electrical field stimulation; mucus secretion is therefore regulated by enteric cholinergic neurons. However, the mediating MR subtype has yet to be determined [8].

5.2. Regulation of smooth muscle function in the GI tract

In rats, the primary mediators of intestinal smooth muscle cholinergic control are the interstitial cells

of Cajal (ICC), which lie between the circular and longitudinal layers of smooth muscle in the GI tract. M2R and M3R are expressed in GI smooth muscle and M1R activation relaxes smooth muscle in M3R-deficient mice [8]. M2R and M3R promote contraction; M2R- and M3R-deficient mice lack GI smooth muscle contraction [17]. M3R is the predominant mediator of smooth muscle contraction; M2R plays a lesser role [13]. Despite activating the same G-protein, M1R and M3R do not share similar effects on GI smooth muscle; M1R relaxes and M3R contracts smooth muscle.

5.3. Fluid transport

Current evidence indicates acetylcholine release at nerve junctions controls fluid transport by enterocytes in intestinal crypts. While vagal-mediated secretion likely involves vasoactive intestinal peptide (VIP), immunohistochemical and agonist-antagonist studies showed intramural nerves may control fluid transport via acetylcholine. Indeed, M1R, M3R, and M5R are expressed on enterocytes in the small bowel with M3R likely being the most important subtype for secretion from colon crypts [8].

5.4. Cell proliferation

Intestinal stem cells renew the epithelium every 2 to 5 days with new cells originating at the base of intestinal crypts. Studies show cholinergic neurons control stem cell proliferation, most likely involving M3R or M5R, not M1R and M4R [8, 18]. M1R, M3R, and M5R are conditional oncogenes when expressed in cells capable of proliferation [19]. Cancers derived from epithelial and endothelial cells express muscarinic receptors; activation of M1R, M3R, and M5R increase cell proliferation [20]. Acetylcholine and secondary bile acids are both muscarinic receptor agonists that stimulate cell proliferation [21]. For most cancers derived from epithelial and endothelial cells, acetylcholine synthesized and released by cancer cells acts as an autocrine and paracrine growth factor [20].

6. Muscarinic receptors in GI cancer

M3R activation increases cancer cell proliferation [8, 22, 23], and activating M1R, M3R, and M5R may inhibit apoptosis [8]. M3R activation promotes cancer in the stomach, colon, pancreas, and liver, often *via* a non-neuronal autocrine or paracrine loop [20]. While M3R may increase cell proliferation, this effect typically requires transactivation of other signaling pathways, such as the epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) cascades [8].

6.1. Colon cancer

In murine models of colon cancer, a non-selective MR agonist, bethanechol, stimulates expression of matrix metalloproteinases (MMPs) important for tumor growth and invasion, as well as other key players in tumorigenesis (EGFR, myc, and cyclin D1) [23]. An autocrine loop involving MR's is important for colon cancer progression; Cheng, *et al.* 2008 showed choline transporter inhibitors attenuated and cholinesterase inhibitors promoted colon neoplasia. In culture, colon cancer cells expressing choline acetyltransferase (ChAT) produced acetylcholine, whereas normal colon enterocytes did not express ChAT [24]. Colon cancer cells over-express M3R. M3R antagonists and choline transport inhibitors attenuated and acetylcholinesterase inhibitors increased the proliferation of colon cancer cells [24]. These proliferative actions are partially due to transactivation of EGF receptors (EGFR). Crosstalk between M3R and EGFR is mediated by activation of matrix metalloproteinase-7 (MMP7), which catalyzes the release of the EGFR ligand HB-EGF [20, 21, 23, 25].

One study using SNU-407 colon cancer cells showed that stimulating muscarinic receptors activates the PI3K-Akt-mTORC1-S6K1 pathway (mTORC1, mammalian target of rapamycin complex 1; S6K1, ribosomal S6 kinase 1) [26], and another study using the same cell line demonstrated phosphorylation of eukaryotic translation initiation factor 4B (eIF4B) *via* the MAPK/ERK1/2 and PKC pathways – not *via* PI3K [26, 27]. Both of these pathways are paramount in MR-mediated protein synthesis, cell proliferation, and survival, but these two studies did not investigate which MR subtype was involved [26, 27]. Another pathway involves crosstalk between M3R and corticotrophin-releasing hormone receptor 2 (CRF2). M3R activation induces synthesis and secretion of urocortin 3 (Ucn3), which activates CRF2 and ultimately initiates endocytosis of E-cadherin and

activation of MMP-2, -7, and -9 *via* a signaling cascade involving Src and ERK1/2. Disruption of the mucosal barrier can increase mucosal inflammation, thereby increasing the risk of colon neoplasia and metastatic potential [28]. MMP-1 expression correlates strongly with adverse outcomes, metastasis, and advanced stage in colon cancer, and activation of M3R leads to upregulation of MMP1 *via* PKC-p38-α (a MAPK) and PKC-Src-EGFR pathways [19]. Additionally, colon cancer cell invasion due to MR agonists is inhibited by preventing the activation of MR and MMP1 [29].

Compared to control mice, M3R-deficient mice treated with azoxymethane to induce colon neoplasia had attenuated cell proliferation and tumor formation [30, 31]. M1R and M1R/M3R dual knockout mice did not exhibit any change in tumor volume or numbers, suggesting M1R deficiency negated the protective effects of M3R deficiency [31]. Most tumors in wild-type, M1R and M1R/M3R double knockout mice were adenocarcinomas, contrasted by similar numbers of adenomas and adenocarcinomas in M3R-deficient mice. These findings suggest M3R deficiency prevents progression of adenomas to adenocarcinomas [31]. In fact, there appears to be differential expression of M3R within different stages of colon cancer, from normal colon epithelium to metastatic cancer. M3R is overexpressed primarily in early stages of colon neoplasia (adenoma and primary adenocarcinoma), but not in lymph node and liver metastases; this suggests overexpression of M3R is more important early in colon neoplasia. As an index of receptor activation in early stages of neoplasia, in contrast to M3R cellular localization on basolateral membranes of normal colon epithelial cells, its expression shifts to the cytoplasm in adenomas and adenocarcinomas [32]. Yet, M3R overexpression in primary colon tumors correlates with metastatic potential. Collectively, these findings suggest M3R expression and signaling are important for colon cancer cell migration and invasion (metastatic potential) but less important once metastases are established.

Interestingly, luminal bile acids are associated with colon cancer *via* activation of M3R and post-receptor EGFR/ERK pathway signaling [21, 30, 33]; selected bile acids functionally bind to M3R [20]. There are several mechanisms whereby bile acid-induced activation of M3R may mediate progression of colon neoplasia: prolonged contact with neoplastic colon epithelial cells, lack of inactivation by cholinesterases, ability to passively or actively traverse cell membranes, loss of polarity, and effects on tight junction permeability [21].

6.2. Gastric cancer

Gastric carcinoma cell lines express M3R, and M3R stimulation can activate the MAPK/ERK1/2 pathway downstream of EGFR or the metastasis-associated in colon cancer-1 (MACC1) oncogene *via* AMP-activated protein kinase (AMPK) [20, 34, 35]. M3R over-expression in gastric cancers correlates with staging and lymph node metastases, and activation of M3R *via* acetylcholine enhances proliferation. Short-hairpin RNA (shRNA) knockdown of M3R decreased cell proliferation, causing G2/M cell cycle arrest, apoptosis with decreased bcl-2, and increased bax expression *in vitro*. M3R expression and activation protect against apoptotic effects of DNA damage, oxidative stress, and mitochondrial inhibition [34, 36]. In gastric cancer xenografts, M3R knockdown suppressed tumorigenesis and promoted apoptosis. As in colon cancer, autocrine signaling may play an important role in increasing gastric cancer proliferation, as gastric cancer cells secrete acetylcholine and express ChAT [34, 36]. Furthermore, treatment with the M3R inhibitor darifenacin attenuated gastric neoplasia even in the absence of stimulation with exogenous ACh, suggesting a constitutive autocrine loop promotes gastric neoplasia [34, 35]. Also similar to colon cancer, it appears that M3R but not M1R promotes cell proliferation, and inhibiting M3R activation attenuates gastric tumor formation [34]. Studies using three different mouse models of gastric cancer showed vagal denervation decreases tumorigenesis *via* suppression of the M3R-Wnt pathway. Suppression of vagus nerve stimulation *via* unilateral vagotomy or botulinum toxin A injection inhibited tumorigenesis on the ipsilateral side; blockade of M3R also decreased tumorigenesis [37, 38].

M3R activation modulates Wnt pathway signaling in the development of gastric tumors [39].

Hayakawa *et al.* found that cholinergic stimulation induced nerve growth factor expression in the stomach, which in turn promoted carcinogenesis. Furthermore, they found that M3R-mediated Wnt activation required the involvement of YES-associated protein (YAP), a downstream target of M3R [39]. YAP participates in activating β-catenin-dependent cancer growth and tumorigenesis [39]. Clearly, a pathway connecting M3R and Wnt signaling is an important mechanism for gastric tumorigenesis.

6.3. Pancreatico-biliary cancer

The endocrine pancreas responds to M3R activation with insulin and glucagon secretion, whereas M1R/M3R activation in exocrine acinar cells stimulates pancreatic enzyme release [20]. M3R is upregulated in pancreatic ductal adenocarcinoma, but is virtually absent in adjacent normal pancreatic tissue. Up-regulation of M3R expression correlates with higher-grade malignancy, lymph node metastasis, and decreased survival, but is not related to neurovascular invasion or T stage. M3R upregulation is observed primarily at the invasive tumor front rather than the tumor core [40]. Overexpression of M3R in cholangiocarcinomas plays an important role in proliferation, differentiation, and tumorigenesis [36]. M3R overexpression in cholangiocarcinoma cells correlates with impaired cell differentiation, hilar distribution, and distant metastases. Interestingly, the MR agonist pilocarpine decreases cholangiocarcinoma cell proliferation in a dose-dependent manner, which surprisingly suggests M3R overexpression in cholangiocarcinoma protects against tumor growth [41].

6.4. Liver cancer

M3R activation may stimulate liver and bile duct regeneration *via* hepatic vagal input. Cassiman *et al.* reported attenuated numbers of hepatocyte and bile duct epithelial progenitors (hepatic progenitor cells) in denervated liver (rats after vagotomy and humans after liver transplant). The likely mediator was M3R, as this was the only MR detected on hepatic progenitor cells by immunohistochemistry [22]. Indeed, in mouse models, M3R deficiency dramatically increased toxin-induced hepatic injury [42]. On the contrary, M1R appears to promote fibrosis, as demonstrated by Rachakonda *et al.* in azoxymethane-treated M1R-deficient mice. M1R deficiency activated anti-oxidant responses, decreased hepatocyte apoptosis, and reduced fibrosis [42]. These data suggest M3R and M1R play contrasting roles in promoting tissue repair, findings likely relevant to their contrasting actions on neoplastic cells.

7. Conclusions

While we have learned a great deal about MR structure and function, much remains to be understood, particularly with respect to organ-specific signaling and function. Clearly, M1R, M3R, and M5R signal differently than M2R and M4R. However, despite M1R and M3R signaling pathway similarity, there is compelling evidence for contradictory actions of their activation in the GI tract. Modulating these functions has potential therapeutic implications; uncovering the basis for these functional differences will require more information regarding MR structure and signaling.

Key amino acid residues important for acetylcholine binding to MR's are conserved in the five MR subtypes [7, 9]. Additional information regarding MR three-dimensional structure obtained from site-directed mutagenesis and X-ray crystallography identify targetable moieties [7, 9]. For example, a vestibule leading to the orthosteric binding site containing amino acids that are not conserved between the five MR subtypes [9] may facilitate entry of ligands into the orthosteric binding site. MR's have at least two allosteric binding sites, and ligands bind to MR's with varying affinities [7]. Bitopic ligands specifically target MR subtypes [10]. These methods of specific MR activation lead to varying downstream effects.

M1R, M3R, and M5R activate $G\alpha_q$, and while the second messengers are largely the same, M1R and M3R activation results in different downstream effects. For example, Gβγ in M3R but not M1R activates PLCβ, and SRF and RhoA are activated by M1R and not M3R [8]. It is not clear what accounts for these differences in M1R and M3R signaling. It is possible that different receptor conformations after binding of subtype-specific allosteric ligands influence downstream signaling [10]. Differential MR subtype expression within the GI tract may impact receptor signaling [8, 15, 16]. Functional differences may arise from activation of different receptors found in unique sites, but

more interesting are the disparate effects on tissues expressing multiple MR subtypes.

Differences in MR structure, signaling, and expression culminate in substantial variation in the overall function of muscarinic receptor subtypes. Within the GI tract, MR's have a wide variety of functions from smooth muscle contraction to secretion to tissue growth and repair. M3R, but not M1R, mediates gastric acid and possibly bicarbonate secretion, and both M1R and M3R regulate pepsinogen secretion [8, 15, 16]. In smooth muscle, M1R and M3R have contradictory effects on contraction: in M3R-deficient mice, M3R contracts and M1R relaxes muscles [8]. M3R activation increases proliferation in multiple GI cancers [20]. Mouse models reveal decreased colon cancer cell proliferation in M3R-deficient mice and in response to treatment with M3R antagonists, whereas M1R-deficient mice exhibited no change in tumor number or size [31]. Similarly, M3R activation promotes gastric cancer cell proliferation *via* the MAPK/ERK1/2 pathway [20]. Similar to the actions of M3R agonists in stimulating the proliferation of neoplastic cells, M3R activation plays a role in liver and bile duct regeneration whereas M1R activation promotes fibrosis [22, 42]. A case report of a patient who developed rapidly progressive colon cancer in the setting of an unresectable pheochromocytoma highlights the importance of further understanding the link between muscarinic ligands and receptors, and GI neoplasia [43].

Specifically how mammalian physiology targets one MR subtype versus another is unclear, though it appears differential expression is one mechanism. Yet, despite similar M1R and M3R structures and signaling pathways, differential expression alone cannot explain why GI smooth muscle and normal and neoplastic epithelial cells respond differently to their activation. Conceptually, access to these cells by other endogenous MR ligands, like bile acids, may differentially activate M1R and M3R; this may provide an evolutionary advantage that explains the expression of otherwise similar receptors.

8. Future directions

Despite great advances in knowledge reviewed here, much remains to be discovered about MR function. While efforts to uncover the three-dimensional structure of MR subtypes have been fruitful, understanding how to target specific MR's *via* differential ligand entry, allosteric binding sites, and bitopic ligands remains a challenge. As M1R and M3R have similar signaling cascades, discovering how these two subtypes are capable of such varying downstream effects may improve understanding of how cancer develops and offer new therapeutic targets. The ability to target specific subtypes may have both diagnostic and therapeutic implications. Because M3R is over-expressed in pancreatic ductal carcinoma, cholangiocarcinoma, gastric cancer, and colon cancer, detecting abnormally high levels of M3R expression may assist early detection of GI cancers. Studies in colon and gastric cancer suggest that M3R is pro-tumorigenic while M1R appears to be tumor suppressive, similar to their opposing effects in liver injury. These findings suggest M3R is involved in tissue repair, which is aberrant in neoplasia. The specific roles of MR subtypes in malignancy married with the ability to target them reliably would open the door for new diagnostic and therapeutic targets in neoplasia, tissue injury, and other GI disorders in which MR signaling plays a key role.

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DISCLAIMER

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts to disclose.

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