Smooth muscle cells, interstitial cells and neurons in the gallbladder (GB): Functional syncytium of electrical rhythmicity and GB motility (Review)

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Abstract. The motility of the gallbladder (GB) involves the storage, concentration and delivery of bile. GB motor functions are controlled by multiple complex factors, such as extrinsic and intrinsic innervation, humoral factors and neuropeptides. GB emptying results from coordinated contractions of the muscular layers of the GB wall. Depolarization of GB smooth muscle (GBSM) depends on the activation of the regular depolarization-repolarization potential, referred to as slow waves (SWs). These rhythmic SWs of GBSM contraction are mediated by several cell types, including smooth muscle cells (SMCs), GB neurons, telocytes (TC) and specialized pacemaker cells called interstitial cells of Cajal (ICC). The present article introduced a new GB motor unit, the SMC-TC-ICC-neuron (STIN) syncytium. In GB, STIN cells provide pacemaker activity, propagation pathways for SWs, transduction of inputs from motor and sensory neurons and mechanosensitivity. The present review provided an overview of STIN cells, mechanisms generating GBSM contractile behavior and GB motility, and discussed alterations of STIN cell function under different disease conditions.

Contents

1. Introduction

The gallbladder (GB), an accessory organ of the gastrointestinal (GI) tract, stores and concentrates most hepatic bile

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Abbreviations: GB, gallbladder; SMC, smooth muscle cell; TC, telocyte; ICC, interstitial cells of Cajal; ICLC, interstitial Cajal-like cells; GSMC, gallbladder smooth muscle cell; STIN, SMC-TC-ICC-neuron; GBSM, gallbladder smooth muscle; SW, slow wave; GI, gastrointestinal; PDGFRα$^+$, platelet-derived growth factor receptor α-positive; SIP, SMC-ICC-PDGFRα$^+$ cell; ENS, enteric nervous system; IHC, immunohistochemical; TEM, transmission electron microscope; α-SMA, α-smooth muscle actin; Ano1, anoctamin 1; NKCC1, Na^+-K^+-Cl- cotransporter; SR, sarcoplasmic reticulum; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; AP, action potential; VDCCs, voltage-dependent Ca^{2+} channels; ERG, ether-a-go-go-related gene; K_{ATP}, ATP-sensitive K^+; BK_{Ca}, large-conductance Ca^{2+}-activated K; CGRP, calcitonin gene-related peptide; RyR, ryanodine-sensitive receptors; CCK, cholecystokinin; CCE, capacitative calcium entry; NSCCs, nonselective cation channels; TRPC, TRP protein family C; IP3R, inositol 1,4,5-trisphosphate receptors; GPCRs, G-protein-coupled receptors; CICR, calcium-induced calcium release; PKC, protein kinase C; ROCK, RhoA/Rho-kinase; PLC, phospholipase C; MR, minute rhythm; CaCCs, Ca^{2+}-activated Cl- channels; NCXs, Na^+/Ca^{2+} exchanger; STICs, spontaneous transient inward currents; 18β-GA, 18β-glycyrrhetinic acid; ChAT, choline acetyltransferase; NPY, neuropeptide Y; SST, somatostatin; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; nNOS, neuronal nitric oxide synthase; TKs, tachykinins; Ach, acetylcholine; NO, nitric oxide; cGMP, 3',5'-cyclic-guanosine monophosphate; CO, carbon monoxide; NT, neurotensin; PYY, peptide YY; TGR5, Takeda GPCR 5; FXR, FGF15/19-farnesoid X receptor; GLP-2, glucagon-like peptide 2; SCF, stem cell factor; AC, acute cholecystitis; AAC, acute acalculous cholec-ystitis; PGs, prostaglandins; ET, endothelin; PGE2, prostaglandin E_2

Key words: gallbladder, smooth muscle cells, interstitial cells of Cajal, telocytes, syncytium

between meals and regulates the outflow of bile into the duodenum postprandial. The human liver normally produces at least 1,000 ml of hepatic bile per day (1). Up to 80% of hepatic bile partitions into the GB, depending on the synergy state of the GB and sphincter of Oddi (2,3). The GB undergoes structural and functional changes, as well as GB dysmotility, in numerous pathological conditions, including gallstone disease, GB polyps and acute acalculous cholecystitis (4-6). Given that GB dysmotility is so prevalent in GB disease, a comprehensive understanding of the neurons and smooth muscles responsible for GB contractile activity is critical.

GI motility patterns, including those of the GB, result from coordinated contractions of the muscular layers of the alimentary canal. Several studies found that interstitial cells of Cajal (ICCs) and platelet-derived growth factor receptor α-positive (PDGFRα$^+$) cells form electrical coupling complexes with smooth muscle cells (SMCs) in the GI tract. Sanders *et al* (7) initially proposed this structure as an SMC-ICC-PDGFRα$^+$ cell (SIP) syncytium. In this functional structure, ICCs act as periodic spontaneous pacemakers to generate a slow wave (SW), which conducts SMCs to drive phasic contractions (8,9). Correspondingly, PDGFRα$^+$ cell excitation causes hyperpolarization of SMCs, leading to muscle relaxation (10,11). Unlike skeletal muscle, there is no classical neuromuscular junction between nerve terminals of the enteric nervous system (ENS) and GI smooth muscle (12). Enteric nerve endings expand to form numerous varicosities containing neurotransmitters (13,14). Subsequently released neurotransmitters diffuse to the adjacent SIP syncytium to regulate GI motility. Although the integrity of the morphological structure and function of SIP syncytium are important for GI physiological function, the functions of SIP syncytium are mainly derived from evaluations of specific SIP cell types.

Previously, telocytes (TCs) were considered interstitial Cajal-like cells (ICLCs) due to the similar morphology under the light microscope and immunohistochemical (IHC) features with ICCs, which were found >100 years ago and considered to be pacemakers for GI motility. Subsequently, it was demonstrated that TCs are not ICLCs, as TCs presented a distinctly different ultrastructure from ICLCs in transmission electron microscopy (TEM) images. To avoid further confusion and to give a precise identity to these cells, in 2010, Popescu and Faussone-Pellegrini (15) coined the term TCs for cells previously referred to as ICLCs. Differences in the TCs' immune phenotypes have been found to be significant in different tissues; by contrast, the ultrastructural differences of TCs are the least evident. Hence, the term TCs was proposed based on the cells' unique TEM features rather than selective immune markers. Subsequently, Vannucchi *et al* (16) clearly indicated that TCs express PDGFRα in the human GI tract. Based on these IHC data, TCs are frequently referred to as PDGFRα$^+$ cells and this definition is commonly used in scientific reports. Of note, as TCs express different IHC markers in different organs and even in different tissues from the same organ, it remains controversial whether TCs and PDGFRα$^+$ cells are the same cell type (17-20). However, in the gut, all cells identified as TCs were double-positive for CD34 and PDGFRα and shared identical ultrastructural features (16,21); therefore, these TCs and PDGFRα$^+$ cells are the same cell type, at least in GI tract. Further research substantiated the existence of TCs in the biliary system, including GB, extrahepatic bile duct, cystic duct, common bile duct and sphincter of Oddi (22).

Current electrophysiological studies of the GI tract are mostly focused on the stomach and intestine. The concept of SIP syncytium was also first demonstrated and proposed in the GI tract (7). Although the histological anatomy and physiological functions of the GB and the stomach or intestine are not identical, they belong to the same myogenic organs of the digestive tract and their physiological functions are both dependent on the movement of their smooth muscles. More importantly, both the expression and distribution of ICCs and TCs have also been demonstrated in myogenic organs such as the GB, ureter and uterus (23-26). Current studies on GB electrophysiology are mainly on SMCs and ICCs (22,27-33). The mechanisms of SMCs in the motor function of the GB have been most thoroughly studied. It is currently believed that ICCs in GB have a regulatory role in the motor function of the GB, but the exact mechanism of regulation remains to be clarified. The study of TCs in the GB is even more limited to histology. However, the regulation of GB motor function is important for benign GB diseases (e.g., cholelithiasis, cholecystitis, GB polyps, GB adenomyosis). In the most recent study by our group, the presence of a unique structure containing ICCs, TCs, SMCs and neurons in the GB has been proved by multiplexed IHC (Fig. 1; for methods see supplementary data). These results indicated that the four cells were in spatial proximity to each other in mouse GB. Furthermore, c-Kit and anoctamin 1 (Ano1) were used to label ICCs, CD34 and PDGFRα to label TCs, Myh11 and Acta2 to label SMCs to analyse the single-cell RNA-sequencing of normal mice (for methods see supplementary data) (34). The results also proved that there were three double-positive cell types (ICCs, TCs and SMCs) for their respective specific molecular markers and they formed their own cell clusters (Fig. 2). All of these results demonstrated that these four types of cells are present and constitute the SMC-TC-ICC-neuron (STIN) syncytium structure in the mouse GB. Based on these findings, the functional complex was proposed as an STIN syncytium (Fig. 3). The present review described various aspects of the morphology, regulation and function of STIN cells in GB and discussed pathological changes of the STIN syncytium in GB disease.

2. Morphology and distribution of STIN cells

Research of GB structure and function is primarily derived from animal studies, particularly guinea pig and mouse models. The identification of individual STIN cells is based on their morphology (Fig. 4; for methods see supplementary data) and immune phenotypes, which are summarized in Table I.

GB smooth muscle cells (GSMCs). Unlike the GI tract, the GB muscle layer only consists of a single layer of SMCs. GB muscle fibers are separated by different amounts of connective tissue and orientated in different directions (35). GSMCs are shuttle-shaped, with abundant thin (actin and calponin) and thick filaments (myosin) in the cell body. Typical binding of actin and myosin results in cross-bridges, which form

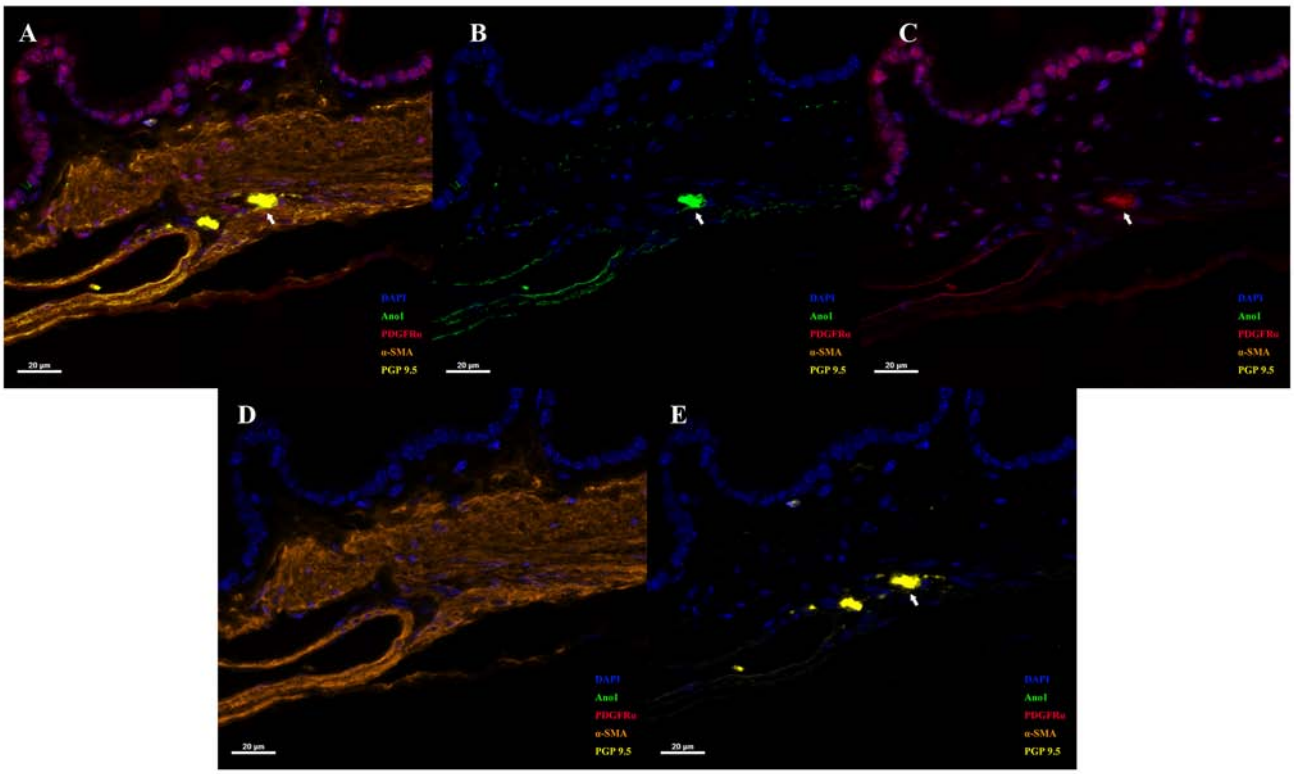

Figure 1. Full-thickness sections of mouse GBs stained by multiplexed immunohistochemistry methods to visualize STIN cells. (A) GB wall containing STIN cells (arrow). Ano1-immunopositive reactivity is displayed in green, PDGFRα-positive in red, α-SMA-positive in orange, PGP 9.5-positive in yellow and cell nuclei were counterstained with DAPI (blue). (B) GB wall containing ICCs marked with Ano1 (arrow). (C) GB wall comprising TCs marked with PDGFRα (arrow). (D) GB wall including GSMCs marked with α-SMA. (E) GB wall containing neurons marked with PGP 9.5 (arrow) (scale bars, 20 μm). STIN, SMCs-TCs-ICCs-neurons; ICCs, interstitial cells of Cajal; TCs, telocytes; GSMCs, gallbladder SMCs; SMCs, smooth muscle cells; PDGFRα, platelet-derived growth factor receptor α; Ano1, anoctamin 1; SMA, smooth muscle actin; GB, gallbladder; PGP 9.5, protein gene product 9.5.

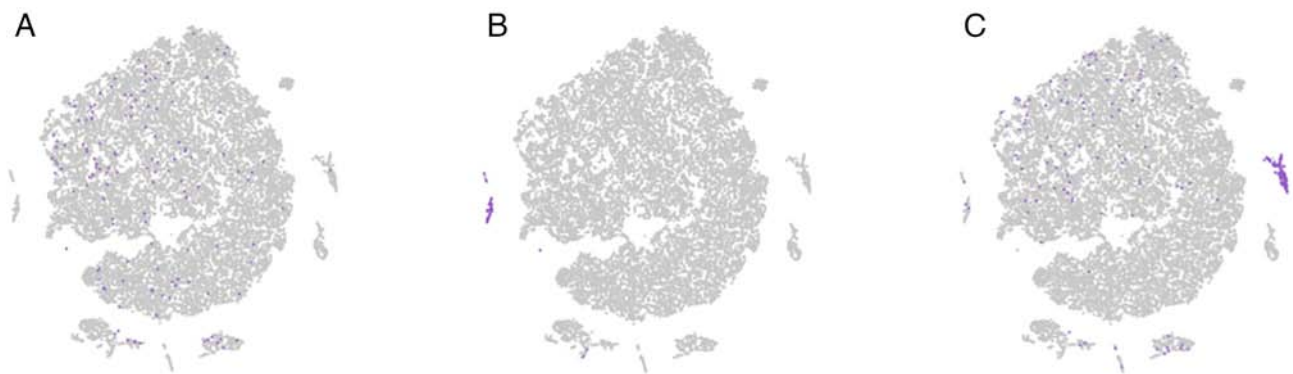

Figure 2. T-distributed stochastic neighbor embedding plot indicating the expression of known marker genes for cell types of normal mouse gallbladder. (A) Interstitial cells of Cajal, c-Kit and anoctamin 1. (B) Telocytes, platelet-derived growth factor receptor α and CD34. (C) Smooth muscle cells, Myh11 and Acta2. The raw data are from the Gene Expression Omnibus dataset GSE179524.

the basic unit of smooth muscle movement (36). α-Smooth muscle actin (α-SMA) is frequently used as a specific marker for smooth muscle (37). Another characteristic structure of GSMCs is the plasma membrane-sarcoplasmic reticulum (SR) junction, which are invaginations of the plasma membrane containing signaling molecules and ion channels (38).

ICCs. Research on GB ICCs began in the 21st century. In 2006, Sun *et al* (39) first confirmed the existence of ICCs in CD1 mouse GB by c-Kit antibody labeling in combination with methylene-blue staining. Later, ICCs were also identified in human extrahepatic bile ducts, where they are more densely aggregated than in the GB (40,41). Light microscopy indicated that ICCs are typically elongated with oval-shaped cell bodies and 1-3 long processes extending from their poles, or exhibit a triangular cell body with several slender lateral branches (42). The fusiform ICCs form a multiple connecting network that is oriented parallel to adjacent muscle fibers in the GB muscularis layer. TEM scanning revealed that ICCs possess large nuclei, a

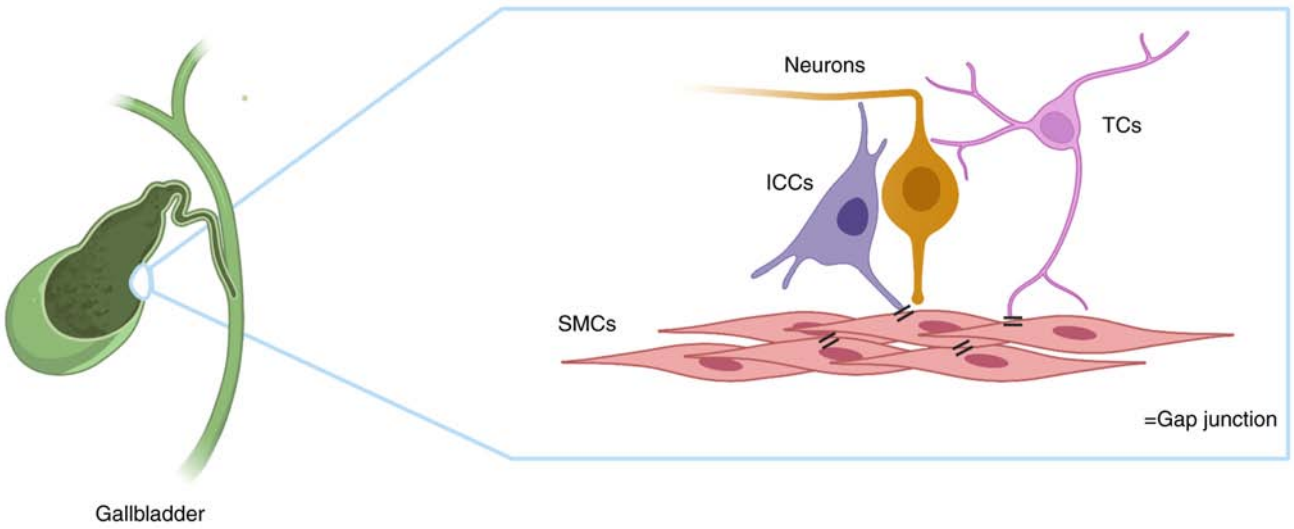

Figure 3. Cellular components of the STIN syncytium. GB neurons, ICCs and TCs are electrically coupled via gap junctions in SMCs, forming the STIN syncytium and providing regulatory control of GB function. In the muscular layer of the GB, ICCs and TCs are closely associated with the terminal processes of GB neurons and express receptors, second-messenger, neurohumoral pathways and ion channels facilitating responses to GB motor neurotransmitters. ICCs are pacemaker cells and generate electrical slow waves. TCs are responsive to purines and participate in the inhibitory neurotransmission of purinergic neurotransmitters. ICC and TCs are electrically coupled to SMCs, which may conduct slow waves to SMCs and regulate the excitability of the musculature in the gallbladder. STIN, SMCs-TCs-ICCs-neurons; ICCs, interstitial cells of Cajal; TCs, telocytes; SMCs, smooth muscle cells; GB, gallbladder.

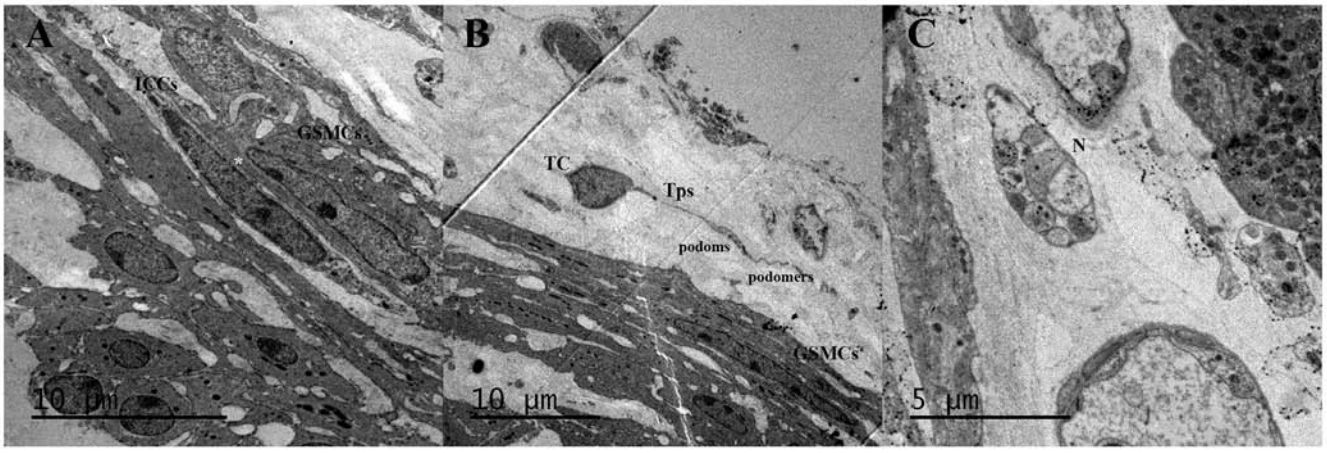

Figure 4. Transmission electron microscopy images of STIN cells in mouse GB. (A) The photographic reconstruction illustrates ICCs rich in mitochondria, smooth endoplasmic reticulum and caveolae observed in muscularis propria. The ICCs form electrical conduction structures with surrounding SMCs through gap junctions (*). (B) TCs have a small oval body, mainly occupied by the nucleus, and are thin and long; the repeatedly folded processes extend beyond the cellular body, which are called Tps. The thin segments are called podomers and the dilated regions podoms (scale bars, 10 μm). (C) The presence of typical GB nerve endings containing abundant synaptic vesicles in the muscular layer of the GB (scale bar, 5 μm). N, neuron; GSMC, GB smooth muscle cell; ICC, interstitial cells of Cajal; TC, telocytes; Tps, telopodes; STIN, SMC-TC-ICC-neurons; GB, gallbladder; SMC, smooth muscle cell.

well-developed smooth endoplasmic reticulum, abundant free perinuclear mitochondria, distinctive caveolae, free ribosomes and intermediate filaments without thick filaments, which are adjacent to SMCs and nerve endings (43). Recently, two identified genes, Ano1 and Na^+-K^+-Cl^- cotransporter (NKCC1), were found to be highly expressed in GB, representing a new and highly selective molecular marker for studying the distribution and fate of ICCs (44,45).

TCs. In 2007, Hinescu *et al* (41) first described TCs in human GB in detail. In the human adult GB, TCs are mostly placed near small vessels in the subepithelial region of the lamina propria and between smooth muscle bundles in the muscularis (46). TEM is considered the most accurate method for identifying TCs (15,26,47). In TEM images, TCs exhibit a variable tiny body with several dichotomously branched, extremely long and thin telopodes. The shape of the cytoplasm varies, including fusiform, pyriform and triangular shapes depending on the number of telopodes, which have a moniliform profile characterized by the alternation of thin tracts with dilations. Hematoxylin and eosin staining revealed long and extremely thin prolongations undetectable by light microscopy. The thin segments are called podomers, while the dilated regions are called podoms. Podoms hold functional units consisting of numerous mitochondria, endoplasmic reticulum and caveolae. CD34 and PDGFRα are considered reliable markers of TCs in the GI tract (48,49). In addition, TCs selectively express the small conductance Ca^{2+}-activated K^+ channel SK3 in the gut,

Table I. Identification of STIN cells in gallbladder.

Author(s), year	Cell types	Location	Morphology	Histochemistry	Special marker	(Refs.)
Sun *et al*, 2006 Pasternak *et al*, 2016 Lavoie *et al*, 2007 Gomez-Pinilla *et al*, 2009 Zhu *et al*, 2016 Pasternak *et al*, 2012 Burns *et al*, 1997 Christensen *et al*, 1992 Ward *et al*, 1990 Mikkelsen *et al*, 1988 Xue *et al*, 1993 Huang *et al*, 2009 Vannucchi *et al*, 2016	ICCs	Muscularis propria layer	Ovoid or triangular, body 1-3 cytoplasmic processes, large nuclei, abundant mitochondria, SER and characteristic caveolae, without thick filaments	Silver chromate stain, MB stain, rhodamine 123 stain, NADH diaphorase stain	c-kit (+), Ano1 (+), NKCC1 (+), CD34 (-), tryptase (-)	(39,42-46, 209-215)
Horowitz *et al*, 1996 Ota *et al*, 2021 Sugai *et al*, 1985 Hartshorne *et al*, 1998	SMCs	Smooth muscle layer	Shuttle-shaped body, numerous thin and thick filaments, plasma membrane-SR junction	Masson stain	α-SMA (+)	(36,37, 216,217)
Popescu *et al*, 2010 Vannucchi *et al*, 2013 Pieri *et al*, 2008 Chen *et al*, 2018 Cretoiu *et al*, 2014 Hinescu *et al*, 2007 Pasternak *et al*, 2012 Peri *et al*, 2013 Lu *et al*, 2018 Yeoh *et al*, 2016 Mnh *et al*, 1998	Telocytes	Muscular layer	Tiny variable body, hallmark Tps with podomers and podoms	MB stain, toluidine blue staining	CD34 (+), SK3 (+), PDGFRα (+)	(15,16,21,22, 26,41,46, 48-50,218)

STIN, smooth muscle cell-telocyte-interstitial cells of Cajal-neuron; SER, smooth endoplasmic reticulum; MB, methylene-blue; NADH, nicotinamide adenine dinucleotide; Ano1, anoctamin 1; NKCC1, Na^+-K^+-Cl^- cotransporter; SR, sarcoplasmic reticulum; α-SMA, α-smooth muscle actin; Tps, telopodes; SK3, small conductance Ca^{2+}-activated K^+ channels; PDGFRα, platelet-derived growth factor receptor α.

which exhibits significant changes in functionality in the context of GI disease (50). TCs always form networks and provide mechanical support in the GI wall. However, the distribution of TCs in GB across various species remains controversial and further study is required to elucidate it.

3. GSMCs: Excitation-contraction coupling units

Depolarization of GSMCs may occur through direct effects of neurotransmitters, hormones and other bioactive regulatory substances on GSMCs, or through the influence of other STIN

cells electrically coupled to GSMCs. In general, contractions are initiated by phosphorylation of myosin light chain (MLC) 20 by Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) or Ca^{2+}-independent myosin light chain phosphatase (MLCP) (51). Phosphorylation of MLC20 facilitates myosin binding to actin, initiating cross-bridge cycling and contraction development.

Electrical properties of GB smooth muscle (GBSM). Intracellular voltage recordings from intact guinea pig GSMCs revealed that characteristic action potentials (APs) have four distinct components: A resting membrane potential of -40 to -50 mV, a rapidly depolarizing (rarely exceeds 0 mV) and transient repolarizing spike, followed by a slowly sustained declining plateau phase, and finally complete repolarization (52).

GSMCs exhibit rhythmic spontaneous APs (0.3 to 0.4 Hz) started by Ca^{2+} entry, mainly through voltage-dependent Ca^{2+} channels (VDCCs) (52). The AP spike results from activation of L-type VDCCs in the absence of a T-type Ca^{2+} current in guinea pig GSMCs (53). The open state of L-type Ca^{2+} channels is regulated by neurotransmitters and drugs (54,55). For instance, L-type Ca^{2+} channel blockers such as nifedipine may abolish spontaneous AP and inhibit GB contraction. L-type channels are critical for proper GSMC function, providing the major source of contractile Ca^{2+}. Depolarization of I_{cat}, a spontaneously active Na^+-mediated nonselective cation channel, was indicated to maintain the resting membrane potential and increase contractility of GBSM, thus stabilizing GB tone (56).

The repolarization of APs is determined by voltage-gated K^+ (K_v) channels and ether-a-go-go-related gene (ERG) K^+ channels (57,58). Potassium reflux via K_v channels is responsible for the repolarization of APs and regulates the contraction of GBSM. These channels demonstrate relatively low sensitivity to aminopyridines but are inhibited by quinine (59). ERG, which encodes a delayed rectifier K^+ channel in GB, contributes to repolarization of both the rapid spike and plateau phase (60). ERG channel blockers prolong repolarization of the plateau phase, increasing basal contractility of GSMCs and their response to receptor activation (57).

Other potassium channels identified in GSMCs include ATP-sensitive K^+ (K_{ATP}) channels and large-conductance Ca^{2+}-activated K (BK_{Ca}) channels. Activation of the K_{ATP} channel causes prolonged hyperpolarization, reducing the frequency of GBSM APs and associated spontaneous GBSM contractions (61). The K_{ATP} channel appears to have a major role in receptor-mediated relaxation of GBSM, as it is responsible for the inhibitory effects of calcitonin gene-related peptide (CGRP) and agonists of H2 receptors for histamine (62,63). In GSMCs, localized Ca^{2+} release events from ryanodine-sensitive receptors (RyR), also called Ca^{2+} sparks, antagonize GSMC excitability by activating BK_{Ca} channels in the nearby plasma membrane (see below) (64). Spontaneous transient activation of BK_{Ca} currents causes transient membrane hyperpolarization of GSMCs that was, in part, inhibited by cholecystokinin (CCK). Additional cellular mechanisms underlying bile acid-induced GBSM relaxation *in vivo* and *in vitro* potentially include activation of BK_{Ca} channels to generate outward currents, thus counteracting contraction (65).

GSMCs also express the SK3 channel. SK3 likely physically associates with ORAI calcium release-activated calcium modulator 1 (Orai1), a plasma membrane protein, to form a signaling complex. Ca^{2+} influx through Orai1 activates SK3 to induce membrane hyperpolarization in GBSM (66). This hyperpolarizing effect of the Orai1-SK3 complex may serve to prevent excessive contraction in response to contractile agonists.

Regulation of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. GBSM excitation-contraction (E-C) coupling is dependent on an increase in the intracellular concentration of Ca^{2+} $[Ca^{2+}]_i$, which is caused by an influx of extracellular Ca^{2+} through VDCCs and/or receptor-operated Ca^{2+} channels, as well as the release of Ca^{2+} from the SR (67). The influx of extracellular Ca^{2+} required for E-C coupling may enter cells through VDCCs, capacitative calcium entry (CCE) or nonselective cation channels (NSCCs).

The predominant class of VDCC in GSMCs is the L-type Ca^{2+} channel. As previously described, global cytosolic $[Ca^{2+}]_i$ is largely dictated by the open state probability of plasmalemmal L-type Ca^{2+} channels, while calcium entry through VDCCs is determined by the cell membrane potential (68). The depletion of intracellular calcium stores activates CCE, a Ca^{2+} entry mechanism at the plasma membrane (69). Thapsigargin, a sarcoplasmic Ca^{2+}-ATPase inhibitor, is able to prevent the accumulation of Ca^{2+} by the SR. Activation of extracellular Ca^{2+}-dependent responses and Ca^{2+} influx by thapsigargin is regarded as evidence in favor of the involvement of CCE (70). Contractile responses to Ca^{2+} re-addition following depletion of SR Ca^{2+} stores with thapsigargin strongly supports CCE as a source of activating Ca^{2+} for GBSM contraction (71). In addition, actin reorganization is proven to participate in the implementation of CCE, supporting a conformational coupling model for this process in naive SMCs (72). NSCCs in GSMCs demonstrate high selectivity for Ca^{2+} over monovalent cations, leading to activation of VDCCs mediating extracellular Ca^{2+} entry and contraction (73). Transient receptor potential (TRP) channels are a large family of NSCCs widely expressed in GSMCs (74). TRPP2 protein belongs to the TRP superfamily and is encoded by the polycystin 2 gene (75). In guinea pig GB muscle strips, knockdown of TRPP2 significantly reduced carbachol-evoked Ca^{2+} release (27). Accumulating evidence demonstrates that TRPP2 not only mediates intracellular Ca^{2+} release, but also regulates extracellular Ca^{2+} influx to enhance $[Ca^{2+}]_i$ (76-78). Furthermore, TRP protein family C (TRPC) is a candidate channel involved in CCE (28). The expression of TRPC protein depends on cytosolic Ca^{2+} levels through activation of Ca^{2+}/calmodulin-dependent kinases and cAMP-response element binding protein.

Calcium influx and release from the SR, also known as intracellular stores, are crucial for GSMC contractility, which primarily depends on increases in $[Ca^{2+}]_i$ (79). Intracellular calcium release from the SR involves the participation of two ligand-gated channel/receptor complexes [inositol 1,4,5-trisphosphate receptors (IP_3R) and RyR] and is regulated by sarcoplasmic/endoplasmic reticulum calcium ATPase (80,81). Calcium release via IP_3R is activated by IP_3, which is generated in response to numerous G-protein-coupled receptors (GPCRs) and tyrosine kinase-linked receptor

activators, including neurotransmitters, hormones and drugs. RyR mediates the rapid release of calcium from intracellular stores into the cytosol, which is essential for numerous cellular functions, including E-C coupling in muscle. Three types of rhythmic spontaneous Ca^{2+} transients were determined by laser confocal imaging of intracellular Ca^{2+} in GBSM whole-mount preparations (31,64,79). Ca^{2+} flashes reflect calcium entry associated with spontaneous APs and simultaneously occur in all GSMCs in the given bundle, although they are asynchronous among nonintersecting bundles. Ca^{2+} waves are rhythmic Ca^{2+} transients propagating within GSMCs that are asynchronous between individual muscle cells in the given bundle; apparently, these waves correspond to subthreshold depolarization of GSMCs. Both flashes and waves triggered by Ca^{2+} release from the SR occur through IP_3 receptors, which is amplified by calcium-induced calcium release (CICR) and VDCCs (82). Superimposed Ca^{2+} waves induce Ca^{2+} flashes, while the summation of spontaneous transient depolarizations results in APs. In the guinea pig GB, rapid Ca^{2+} transients occur simultaneously in all the GSMCs of a given bundle, but without synchronization between muscle bundles (38). Of note, synchronous Ca^{2+} flashes occur among smooth muscle bundles in the presence of CCK or muscarinic agonists. These findings indicate that the net tone in the GB originates from asynchronous, multifocal contractions of bundles throughout the tissue wall, while synchronous electrical rhythms occurring in all muscle bundles may contribute to GB emptying. Therefore, flashes and waves are critical in maintaining the basal tone and neurohormonal-induced stimulation of GB motility and emptying. Ca^{2+} release from intracellular stores not only induces contraction, it also induces relaxation. Ca^{2+} sparks are another type of focal, nonpropagating calcium transients caused by the coordinated opening of a cluster of RyR. In GB, Ca^{2+} sparks do not lead to any elevation in global $[Ca^{2+}]_i$. Instead, transient localized $[Ca^{2+}]_i$ elevations through opening of BK_{Ca} channels cause SMC hyperpolarization and relaxation (64).

Ca^{2+}-independent MLCP pathway. GSMC contraction is also regulated by Ca^{2+}-independent mechanisms via protein kinase C (PKC)/CPI-17 or RhoA/Rho-kinase (ROCK)-mediated pathways. The regulation of MLC phosphorylation by MLCK causes SMC contraction, whereas inhibition of MLCP may enhance the extent of MLC phosphorylation and SMC contraction and increase Ca^{2+} sensitivity, a phenomenon known as Ca^{2+} sensitization (83). In the classical PKC/CPI-17 pathway, G proteins cause activation of phospholipase C (PLC), diacylglycerol output and activation of PKC. PKC phosphorylates CPI-17, an inhibitor of MLCP activity, resulting in GBSM contraction (84,85). ROCK also regulates GSMC contraction by regulating the Ca^{2+} sensitization mechanism. Contractions induced by carbachol and CCK are mediated by GPCR muscarinic M_3 receptors and CCK_1 receptors in guinea-pig GBSM (86,87). The selective ROCK inhibitor Y-27632 significantly inhibited GBSM contractions evoked by carbachol and CCK *in vitro* (30). In human GB, Y-27632 markedly reduced 5-hydroxytryptamine, neurokinin A and KCl-induced contractions (88). The results of these studies indicate that a RhoA/ROCK-mediated pathway has a role in the regulation of GSMCs.

4. ICCs: Pacemaker of SWs

GB SWs were first recorded by Romański (89) through electromyography. However, the signal of SWs was not always observed and variable in frequency and amplitude. The minute rhythm (MR), another rhythmic activity, consisted of a series of spike potentials recurring at minute intervals (90). The MR has been proven to regularly occur in the entire ovine small intestine and GB, which is controlled by both nicotinic and muscarinic receptor subtypes (91). However, it appears improbable that the MR spike bursts significantly contribute to the enhancement of GB filling or evacuation. Thus, the role of the MR in GB may be to maintain normal tension of the GB wall during the fasting period. Loss of ICCs is associated with a lack of SW activity of GB and the GI tract (92,93). However, the relationship between MR and ICCs requires further study.

Conduction of SWs and regulation of GSMCs. ICCs have an important role in producing and propagating rhythmic electrical activity and GB motility. Isolated ICCs display spontaneous electrical rhythmicity similar to the electrical activity of intact muscles. In fact, electrical coordination between regions of SMCs must occur through the integrity of ICC networks due to the lack of ion channels to regenerate or actively propagate SWs (43,94). In GBSMs, SWs may also be recorded from SMCs due to electrical coupling with ICCs. The function of SWs is to change the membrane potential from a state of low open probability for VDCCs to depolarization, which means APs, when there is an increased probability of associated ionic channel opening (9). A Ca^{2+} imaging study by Lavoie *et al* (43) indicated that the intensity of fluo-4 fluorescence in ICCs was higher than that of the surrounding GSMCs, while rhythmic Ca^{2+} flashes were synchronized in any given GBSM bundle and associated with ICCs. More importantly, gap junction blockers may eliminate or markedly disrupt spontaneous rhythmic Ca^{2+} flashes in GBSM, but persist in ICCs, whereas the selective Kit tyrosine kinase inhibitor imatinib mesylate disrupted or abolished APs and Ca^{2+} flashes in both cell types, as well as associated GBSM contractions. These results demonstrate that the spontaneous rhythmic activity detected in GBSM, which corresponds to smooth muscle bundle contractions, is generated by specialized ICCs and not an intrinsic property of GSMCs. Taken together, ICCs conduct pacemaker SWs into neighboring GSMCs, causing membrane depolarization, opening of the VDCC, intracellular Ca^{2+} release and activation of the contractile apparatus of GB. To date, no specific 'pacing region' has been identified in the GB.

Pacemaker mechanism of ICCs. ICCs serve as pacemaker cells and express a specialized apparatus that includes Ca^{2+}-activated Cl^- channels (CaCCs), T-type voltage-dependent Ca^{2+} channels, NSCCs, NKCC1, inward rectifier K^+ channels and Na^+/Ca^{2+} exchanger (NCXs). SWs recorded from ICCs have fast upstroke depolarizations with large amplitudes and a sustained plateau potential.

SWs in ICCs are mediated by activation of Ano1 channels and NSCCs. ICC depolarization depends upon activation of CaCCs encoded by the ANO1 gene, such that loss or block

of Ano1 abolishes the electrical activity of SWs in intact smooth muscles (95). Periodic activation of Ano1 channel clusters generates spontaneous transient inward currents (STICs) and subsequently initiates coordinated activation of CaCCs that summates to cause the depolarization responses known as SWs (96). The calcium entry from RyR and IP3R of ICCs during CICR appears to be the signal coupled to activation of CaCC, as these channels are sensitive to $[Ca^{2+}]_i$ (97). Of note, research on cultured ICCs indicated that NSCCs, not CaCCs, generated the inward current responsible for SWs (95,96,98,99). This may be explained by rapid loss of Ano1 expression in cell culture and alteration of the auto-rhythmicity retained by ICCs compared with the pacemaker activity of cells *in situ*. Unitary potentials, which are small irregular noisy fluctuations in membrane potential, may be the primary pacemaker activity that underlies SWs. These electric events were insensitive to concentrations of niflumic acid (the inhibitor of CaCC) that blocked SWs (99). The Ca^{2+}-inhibited NSCC-activated STICs observed from isolated ICCs may be responsible for unitary potentials (95). Accordingly, NSCC may contribute to the pacemaker current and generation of electrical SWs in GI smooth muscles. T-type Ca^{2+} channels coordinate Ca^{2+} release from stores in ICCs, thus controlling the openings of Ano1 channels responsible for SW currents (100). The mechanism of SW propagation in tissues has been explored by using muscle strips and partitioned recording chambers. Reduced extracellular Ca^{2+} or antagonists of T-type Ca^{2+} channels inhibit SW upstroke depolarization velocity and propagation (101). These results suggest that SWs propagate through the ICC network by a voltage-dependent mechanism that relies on activation of T-type Ca^{2+} channels (38). ICCs have been demonstrated to express genes encoding inward rectifying K^+ channels, and this inwardly rectifying conductance contributes to the regulation of resting potentials and excitability of SMCs (102).

The plateau component of SWs was dependent on the Cl^- current through CaCCs, while the activation of Ano1 channels results in efflux of Cl^- during SWs (103). Thereby, a mechanism must exist for the recovery of Cl^- loss. IHC confirmed that NKCC1 is expressed at high levels in ICCs (104). Inhibition of NKCC1 with bumetanide and gene knockout of NKCC1 both diminished the plateau component of SWs without directly affecting Ano1 or T-type Ca^{2+} channels (45,105). In isolated GB ICCs, inhibitors of mitochondrial NKCC1 also abolished spontaneous rhythmic activity, suggesting that NKCC1 may have an important role in maintaining the Cl^- gradient supporting the driving force for the inward current mediated by Ano1 (106). Furthermore, NKCC1 may elongate the plateau phase by activation of reverse-mode NCX. NCX, an ion transport protein, extrudes Ca^{2+} in parallel with the plasma membrane ATP-driven Ca^{2+} pump (107). NCX has dynamic features in the SW cycle, in which Ca^{2+} exit helps to maintain the basal $[Ca^{2+}]_i$ between SWs and deactivate Ano1 channels at the end of the plateau; furthermore, Ca^{2+} entry sustains the activation of Ano1 channels during the plateau phase of SWs (108). The longevity of the plateau phase is related to the duration of time that NCX remains in Ca^{2+} entry mode. However, the underlying molecular mechanisms of SWs in GB ICCs remain to be further elucidated.

5. Telocytes: Purinergic inhibitory neurotransmission bridge

In the GI tract, TCs are electrically coupled with ICCs and SMCs, and in close apposition with enteric motor neuron varicosities (10). IHC studies indicated that TCs highly express gap junction genes, as well as SK3 and purinergic P2Y1 receptors (48,109,110). *In vitro*, isolated TCs respond to P1Y1 agonists by activating SK3 channels (111). Purinergic compounds, such as ATP, ADP and β-NAD, elicited large-amplitude outward potassium currents in TCs that were blocked by P2Y1 receptor antagonists and SK3 channel antagonists. This outward current causes hyperpolarization of SMCs, ultimately leading to GI relaxation. Further research suggested that P2Y1 receptors mediate purinergic inhibitory responses in GI muscles, as this relaxation reaction was absent in P2Y1-knockout mice (112). These findings indicate direct innervation of TCs by motor neurons. TCs are the primary targets for purinergic neurotransmitters in inhibitory neurotransmission. The high expression of P2Y1 and SK3 in TCs has a key role in purinergic inhibitory regulation.

SMCs also express SK3 and purinergic receptors (113). However, a previous study indicated that SMCs, stimulated directly with purine agonists, exhibit either no response or small inward currents and depolarization (114). Another study suggested that the gap junction uncoupler 18β-glycyrrhetinic acid blocked neural responses in SMCs, but not in nerve processes or TCs (115). These data indicate that the large-amplitude hyperpolarization responses elicited in GI muscles by purine neurotransmission are more likely to be meditated by TCs than SMCs. Hyperpolarization responses are conducted to SMCs via gap junctions. No evidence suggests that TCs may either generate or regenerate SWs. However, there are no electrophysiological studies on GB TCs. Thus, the role of TCs in the regulation of GB motor function requires further investigation.

6. GB neurobiology

GB relaxation and contraction are primarily myogenic, but the GB plexus has a major role in monitoring the state of the GB, in turn controlling its volume, strength of contractions and bile secretion through ENS reflexes (116,117). The innervation of GB consists of the serosal plexus, muscular plexus and mucosal plexus (118). The most prominent network is the serosal plexus with small, irregularly shaped ganglia connected by bundles of unmyelinated axons (119-121). The serosal plexus is connected to nerve bundles that parallel the extensive vascular distribution in the same layer. However, in humans, the muscular plexus is prominent and does not contain ganglia (122-124). Unlike GI neurons, all GB neurons are cholinergic and immunoreactive for choline acetyltransferase (ChAT) (118). The guinea pig is the most comprehensively studied species in this field. According to chemical coding patterns, the overall population of cholinergic neurons may be divided into two distinct subtypes (125,126): The first type (accounting for >80% of neurons) is immunoreactive for substance P, neuropeptide Y (NPY), somatostatin (SST) and orphanin FQ, and ChAT; the other one is immunoreactive for vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating

polypeptide (PACAP) and neuronal nitric oxide synthase (nNOS). In humans, most GB neurons express VIP, NPY, SST and PACAP, and also contain tachykinins (TKs) (123,127,128). Electrophysiological research of GB neurons indicates they rarely exhibit spontaneous APs and must be driven by extrinsic inputs to release neuroactive compounds onto their target cells, mostly GSMCs (129,130). ICCs and TCs are also tightly associated with excitatory and inhibitory motor neurons in the GB, and connected electrically to GSMCs. Several studies have indicated that numerous neurotransmitters and hormones may regulate GB motility (Table II).

Excitatory transmitters and hormones. GB neurons are relatively unexcitable, driven instead by vagal inputs and modulated by hormones, peptides released from sensory fibers, and inflammatory mediators (118).

CCK, an important gut hormone secreted by enteroendocrine I-cells of the upper small intestine, mainly exerts its physiological functions in GB through the activation of GPCRs identified as CCK_1 receptors. CCK_1 receptors have been identified in both GSMCs and ICCs of human and guinea pig GB and are responsible for the stimulation of contraction (131,132). Previous electrophysiological studies of the GB demonstrated that CCK has presynaptic facilitatory effects within neural ganglia to increase acetylcholine (ACh) release from vagal terminals onto GB neurons, and also stimulates vagal afferent nerve fibers in the duodenum, thus increasing stimulation of vagal preganglionic neurons (133). Furthermore, CCK induces a decrease in resistance of the sphincter of Oddi, a determinant of GB emptying (134). In brief, CCK coordinates the pressure gradient in the biliary system by promoting GB emptying and relaxing the sphincter of Oddi, ultimately facilitating bile evacuation during the feeding period.

Co-expression of TKs with ACh in GB neurons indicates that these factors may act together to promote GB emptying following afferent nerve stimulation (130). M_3 receptors are the major muscarinic receptor in GB and M_4 receptors appear to enhance carbamylcholine-induced contractility of GBSM (135). Release of ACh from neurons results in the contraction of GBSM via activation of M_3 receptors on GSMCs. Activation of M_3 receptors leads to phosphatidylinositol hydrolysis by the G protein-coupled PLC pathway and inhibits cAMP accumulation (136). In human GB, M_3 muscarinic receptors are mainly regulated by voltage-gated Ca^{2+} channels and ROCK (137). The TKs contract the guinea pig GB *in vivo* and *in vitro* by acting on NK2 receptors (138). TKs-induced muscle contraction involves activation of PKC, for which stimulation of inositol phospholipid hydrolysis was associated with the state of NK2 receptors (139).

Bradykinins and their receptors (B_1 and B_2) are potent mediators of inflammation, smooth muscle contraction and nociception. In human and guinea pig GB, bradykinin has been demonstrated to evoke a robust contraction via B_2 receptor activation (140,141). Bradykinin-induced contraction of GBSM *in vitro* relies on the synthesis of prostanoids, whose activation evokes inflammatory responses either by direct stimulation of effector cells or through the release of other mediators, including prostanoid, NO and peptide neurotransmitters. By contrast, B_1 receptors are rarely expressed in normal GB and their upregulation most probably depends on the inflammatory state of the tissue. Activation of B_1 receptors has been related to the maintenance of chronic pain and inflammation (142). Thus, the kinins system has a major role in evoking contraction in normal and, in particular, inflamed GB by stimulating both B_1 and B_2 receptors.

The physiological source of ATP in GB remains elusive and it is possible that ATP functions as a neurotransmitter (143). ATP is known to act on two different classes of P2 receptors, P2X ion channels and G-protein-coupled P2Y receptors (144). The dominant role of G protein-coupled $P2Y_4$ receptors in ATP-induced contraction has been confirmed in guinea pigs. ATP likely stimulates $P2Y_4$ receptors within GSMCs and, in turn, prostanoid production via cyclooxygenase-1, leading to increased excitability of GBSM (145). In the guinea pig, high levels of $P2X_2$ and $P2X_3$ expression are found in sensory fibers of the paravascular plexus. Double labelling IHC revealed that $P2X_2$ and $P2X_3$-immunoreactive neurons were also immunoreactive for VIP, CGRP and nNOS (146).

Inhibitory transmitters and hormones. Neurotransmitters that have an inhibitory effect on GBSM include calcitonin, CGRP, VIP, PACAP and NO. Humoral factors that relax the GB include pancreatic polypeptide (PP), SST and fibroblast growth factor (FGF)15 in mice or FGF19 in humans.

CGRP may induce concentration-dependent relaxation of GB *in vivo*, but has no effect on resting GB pressure (147). CGRP did not affect the release of CCK and the excitatory effect of CGRP was completely abolished by pretreatment with atropine. This implies that the site where CGRP activates contractile activity is on intramural cholinergic neurons rather than GSMCs. This relaxation is primarily due to the opening of K_{ATP} channels, as well as the cAMP pathway (62,148). The increased levels of NO observed when CGRP was present suggest NO is also involved in the CGRP-induced relaxation response (149). NO has been proposed to serve as a neurotransmitter in non-adrenergic non-cholinergic nerves. Synthesized by nNOS, NO stimulates soluble guanylate cyclase enzyme in GSMCs, leading to the formation of 3',5'-cyclic-guanosine monophosphate (cGMP), which mediates GB relaxation (150). Endogenous carbon monoxide (CO) produced in the GB may act as a mediator in relaxation reactions by increasing cGMP levels (151). Of note, despite persistent nNOS expression in heme oxygenase 2-knockout mice, their responses to stimulation are nearly abolished, whereas exogenous CO restored normal responses, indicating that NO does not function in the absence of CO generation (152).

VIP and PACAP are members of a VIP-secretin-glucagon superfamily of structurally related peptide hormones that exert their physiological actions through three GPCRs: PAC_1, $VPAC_1$ and $VPAC_2$ (153). VIP is thought to work as a neurotransmitter of vagus nerve terminals, which relaxes GBSM, decreases GB pressure and inhibits CCK-induced contractions (127,154,155). PACAP was able to produce both contraction and relaxation of CCK-induced GB preparations according to the resting GB tone (156). The dual effects of PACAP are likely mediated through a different type of receptor. Specifically, PACAP induces GB contraction through binding of PAC_1 receptors in unstimulated strips, while the relaxant effect of PACAP in CCK-contracted muscle strips appears to be directly mediated by GSMCs through $VPAC_2$ receptors (157).

Table II. Neuroactive compounds in STIN syncytium.

A, Excitatory compounds

Author(s), year	Neuroactive compounds	Receptors/ synthetase	Mechanisms	Effectors	(Refs.)
Yu *et al*, 1998 Schjoldager *et al*, 1989 Xu *et al*, 2008 Mawe *et al*, 1994 Behar *et al*, 1987 Cawston *et al*, 2010	CCK	CCK_1 receptors	GPCRs-PLC pathway; induction of ACh release	Facilitation of bile evacuation by coordinating the pressure gradient in the biliary system	(86,131-134, 219)
Stengel *et al*, 2002	ACh	M_2 and M_3 receptors	GPCRs-PLC pathway;	Activation of M_2 and M_3 receptors resulting in the contraction of the GB	(135-137)
Takahashi *et al*, 1994			RhoA/ROCK pathway		
Lee *et al*, 2013		M_4 receptors		M_4 receptors appear to be required for optimal functioning of M_2 and M_3 receptor	
Patacchini *et al*, 1992 Yau *et al*, 1990	TKs	NK_2 receptors	PLC-PKC pathway	Excitation GSMCs	(138,139)
O'Riordan *et al*, 2001	BKs	B_1 receptors	Receptor upregulation	Upregulation under inflammatory pathological states	(140-142)
Trevisani *et al*, 2003 Andre *et al*, 2008		B_2 receptors	COX-1	Induction of PE synthesis	
Takahashi *et al*, 1987 Bartoo *et al*, 2008	ATP	$P2Y_4$ channels	COX-1	Induction of PE synthesis	(143,145)
Greaves *et al*, 2000 Parkman *et al*, 1997	PACAP	PAC_1 receptors	PLC-PKC pathway	Excitation of resting state of the GB	(156,157)

B, Inhibitory compounds

Author(s), year	Neuroactive compounds	Receptors/ synthetase	Mechanisms	Effectors	(Refs.)
Zhang *et al*, 1994 Kline *et al*, 1997 Kline *et al*, 1994 Zhang *et al*, 1994	CGRP	CGRP receptors	cGMP-PKG pathway	Hyperpolarization of GSMCs via K_{ATP} channel; Relaxation GSMCs	(62,148, 149,220)

Table II. Continued.

B, Inhibitory compounds

Author(s), year	Neuroactive compounds	Receptors/ synthetase	Mechanisms	Effectors	(Refs.)
				via dephosphorylation of MLC; Induction of NO release of GB neurons	
Gultekin *et al*, 2006 Luman *et al*, 1998	NO	nNOS	cGMP-PKG pathway	Relaxation of GSMCs via dephosphorylation of MLC	(150,221)
Alcón *et al*, 2001 Xue *et al*, 2000 Farrugia *et al*, 1998	CO	HO-2	cGMP-PKG pathway; Interaction with NO as cotransmitters	Relaxation of GSMCs via dephosphorylation of MLC; CO may enhance nNOS catalytic activity or facilitate NO release from GB neurons	(151,152, 222)
Harmar *et al*, 2012 Pálvölgyi *et al*, 2005 Pang *et al*, 1998 Greaves *et al*, 2000 Parkman *et al*, 1997 Zhang *et al*, 2014 Morales *et al*, 2004 Bitar *et al*, 1982	VIP	$VPAC_1$ and $VPAC_2$ receptors	cAMP-PKA pathway; Interaction with nNOS	Hyperpolarization of GSMCs via K_{ATP} channel; Inhibition of the CCK-induced contraction, while increasing the tension of the sphincter of Oddi	(153-157, 223-225)
Harmar *et al*, 2012 Pang *et al*, 1998 Greaves *et al*, 2000 Parkman *et al*, 1997 Morales *et al*, 2004	PACAP	$VPAC_2$ receptors	cAMP-PKA pathway	Hyperpolarization of GSMCs via K_{ATP} channel	(153,155-157,224)
Lavoie *et al*, 2010 Jain *et al*, 2012 Yusta *et al*, 2017 Kliewer *et al*, 2015	BAs	FGF15/19	FGF15/19-FXR pathway	Partly rely on the cAMP-PKA pathway to relax GSMCs	(172-175, 226)

Table II. Continued.

B, Inhibitory compounds

Author(s), year	Neuroactive compounds	Receptors/ synthetase	Mechanisms	Effectors	(Refs.)
Choi *et al*, 2006		TGR5 receptors	cAMP-PKA pathway	Hyperpolarization of GSMCs via K_{ATP} channel	
		GLP-2 receptors	TGR5-GLP-2 pathway	Binding of TGR5 in L cells and promotion of GLP-2 release	
Vu *et al*, 2001 Maselli *et al*, 1999 Yamasaki *et al*, 1995 Kaczmarek *et al*, 2010	SST	SST receptor 2 and SST receptor 5	/	Reduction of CCK secretion as well as ACh release; Inhibition of intrinsic excitatory innervation of GB	(167,168, 227,228)
Mawe *et al*, 2001 Holzer *et al*, 2012 Chen *et al*, 1998	NPY	Y_1 and Y_2 receptors	/	Sympathetic nerves pathway	(125,161, 162)
Holzer *et al*, 2012 McGowan *et al*, 2004 Hoentjen *et al*, 2001	PYY	Y_2 receptors	/	Inhibition of vagal-cholinergic pathway	(161,163, 164)
Holzer *et al*, 2012 Hazelwood *et al*, 1993 Kojima *et al*, 2007	PP	Y_4 receptors	/	Influence on the afferent hepatic vagus	(161,165, 165)

STIN, SMC-telocyte-interstitial cells of Cajal-neuron; CCK, cholecystokinin; GPCRs, G-protein-coupled receptors; PLC, phospholipase C; ACh, acetylcholine; M, muscarinic; ROCK, Rho-kinase; TKs, tachykinins; NK, neurokinin; PKC, protein kinase C; GB, gallbladder; GSMCs, GB smooth muscle cells; BKs, bradykinins; PE, prostaglandin E; PACAP, pituitary adenylate cyclase-activating polypeptide; CGRP, calcitonin gene-related peptide; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; K_{ATP}, ATP-sensitive K^+ channel; MLC, myosin light chain; NO, nitric oxide; CO, carbon monoxide; HO, heme oxygenase; nNOS, neuronal nitric oxide synthase; VIP, vasoactive intestinal polypeptide; PKA, protein kinase A; BAs, bile acids; FGF, fibroblast growth factor; TGR5, Takeda GPCR 5; GLP, glucagon-like peptide; SST, somatostatin; NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide.

Other gut hormones, such as the NPY family, SST and neurotensin (NT), also enhance GB relaxation (158-160). However, it remains elusive whether these hormones regulate GB tone through direct effects on ICCs, GSMCs and TCs, as there is no direct evidence that their respective specific receptors are expressed in GB. The NPY family contains biological active peptides of the gut-brain axis, including NPY, peptide YY (PYY) and PP (161). In guinea pigs, sympathetic postganglionic nerves are immunoreactive for NPY (125). These nerves likely represent the principal source of inhibitory neural input to the GB, leading to a decline of GB tone (162). PYY and PP are almost exclusively expressed in the GI tract. PYY is a GI peptide secreted from endocrine L cells localized in the distal small intestine, colon and rectum (163). PYY was able to abolish the cephalic phase of postprandial GB emptying and probably acts via vagal-dependent rather than

CCK-dependent pathways (164). PP is postprandially secreted from the pancreas, in which it is synthesized by endocrine F cells of the pancreatic islets. Similar to PP, PYY infusion results in increased volume and filling of the GB (165). Circulating PP binds to Y4 receptors in the dorsal vagal complex and affects the hepatic vagal afferent, leading to the inhibition of GB contraction and pancreatic exocrine secretion (166). SST, a peptide with potent inhibitory actions on GB contraction, enhances GB relaxation and reduces plasma excitatory gut hormone (ACh and CCK) secretion during the late postprandial phase (167). SST at a pathological concentration was able to inhibit the GB motor response to intrinsic excitatory innervation *in vitro* (168). NT, a peptide consisting of 13 amino acids, may either stimulate or inhibit GB motility, depending on the dose and species (169). NT induced a dose-dependent contraction of isolated GB of guinea pigs, and these contractile effects resulted from the excitement of cholinergic neurons in the myenteric plexus of GB (170). However, intravenous infusion of NT caused relaxation of the GB in humans (160). Of note, this contractile response was not observed *in vitro* (171).

Recently, bile acids (BAs) have been recognized as signaling molecules capable of regulating GB filling through two different mechanisms: The BAs-Takeda GPCR 5 (TGR5) pathway and the FGF15/19-farnesoid X receptor (FXR) pathway. TGR5 expression was identified in both enteroendocrine L cells and GSMCs (172,173). First, separate BAs were able to directly bind TGR5 in GSMCs, promoting GB filling. In addition, BAs in the intestinal lumen stimulated TGR5 on enteroendocrine L cells, which released glucagon-like peptide 2 (GLP-2) that subsequently activated GLP-2 receptors on GSMCs, ultimately mediating relaxation (174). BAs also activate the FXR expressed by enterocytes, thereby mediating the synthesis and release of FGF15/19 into the blood and subsequent stimulation of FGF receptors on GSMCs, inducing GB relaxation (175). Of note, activation of FXR of enteroendocrine L cells may inhibit GLP-2 release, and this effect may antagonize BA-induced relaxation of GB under certain circumstances.

7. STIN syncytium and the pathophysiology of GB diseases

Cholelithiasis. Cholelithiasis is a highly prevalent digestive system disorder with high socioeconomic costs worldwide (176). In China, the incidence of cholelithiasis is nearly 8-10% and has been gradually increasing in recent years (177). Depending on individual composition and location, gallstones contain >90% cholesterol and the remaining material is black or brown pigment stones (4).

The loss of ICCs results in GB dysmotility in patients with cholesterol or pigment stones, as well as animal models of gallstone disease (33,178). Hypercholesterolemia is an independent risk factor for cholelithiasis, as it may increase biliary cholesterol concentrations, consequently leading to bile crystallization and, ultimately, gallstone formation (179,180). More importantly, cholesterol accumulation strongly damaged the density and ultrastructure of GB ICCs by inhibiting the stem cell factor (SCF)/c-Kit pathway, and disrupted membrane receptor functions of STIN cells, particularly CCK1 receptors (181-183). Due to impaired CCK-induced emptying, the resulting GB stasis provides a microenvironment for excess cholesterol to remain in the lumen; in turn, the elevated cholesterol content further impairs GB emptying (184). During the chronic pathogenesis of cholelithiasis, cholesterol induces an oxidative stress response with characteristic concentration dependence, resulting in inhibited proliferation and continuous apoptosis of GB ICCs (185,186). *In vitro* studies suggested that cholesterol decreases Ca^{2+} channel function and the fluidity of caveolar regions, causing sequestration of excitatory receptors to support reduced binding of agonists in affected GBSM (187,188). High cholesterol diets also significantly inhibit ROCK expression in GMSCs, leading to the promotion of gallstone formation (189). Therefore, enhancement of ROCK expression in GSMCs may be a novel strategy for the prevention and treatment of cholelithiasis.

Hydrophobic bile salts decrease GB contractility, an effect directly related to the hydrophobicity of bile salt (190,191). Hydrophobic bile salts hyperpolarize GSMCs by binding to the GPCR GPBAR1 (also known as TGR5) and activating cAMP-mediated opening of K_{ATP} channels, eventually disrupting GBSM function (172). The reduction in the number of ICCs may be a consequence of the toxicity of hydrophobic bile salts, while other bile components (such as glycocholic and taurocholic acids) may exert protective effects on ICCs (192). However, whether BAs are able to directly injure ICCs requires further study.

Patients with gallstones display abnormalities of the GB neural network. Specifically, IHC of GB with gallstones featured a significant decrease of neurons and enteric glial cells compared with that of GB without gallstones, while calretinin-positive neurons were not different between the two groups of patients (193). Calretinin has been identified as a marker of Dogiel type II gut neurons, which appear to behave as mechanosensors. Thus, these findings support the hypothesis that GB wall mechanics remain intact in patients with or without gallstones, whereas GB motility is impaired.

Acute cholecystitis. Gallstones are responsible for 90-95% of cases of acute cholecystitis (AC), while ~5-10% of patients exhibit acute acalculous cholecystitis (5,194). The pathogenesis of AC is complex and multifactorial, but GB dysmotility is the most critical pathogenic factor, as it may cause GB ischemia, cholestasis and secondary bacterial infection.

Inflammation induces alterations of Ca^{2+} sensitization observed in AC by desensitizing Ca^{2+} pools and impairing the functional status of plasma membrane Ca^{2+} channels (195). Inflammation also reduces the expression of contractile proteins, such as F-actin in GSMCs, which may be responsible for the observed reduction in sensitivity of E-C coupling (195). Inhibition of MLCP mediated by the RhoA/ROCK pathway may also be responsible for the impairment of the contractile response (84). Hydrophobic bile salts may enhance inflammatory processes, as they may diffuse through the mucosa and affect the generation of reactive oxygen species (ROS) by GBSM, either by direct action on GSMCs or increasing numbers of inflammatory cells in the GB wall (196).

Like other inflammatory processes, AC involves the release of inflammatory factors, including prostaglandins (PGs), ROS, histamine and endothelin (ET). Early studies of AC patients demonstrated that both the mucosa and muscularis of GB produce high levels of PGE_2 and the severity of inflammation

was associated with the concentration of PGE_2 (197). Symptoms of AC are significantly reduced during the first 24 h by the cyclooxygenase inhibitor indomethacin (198). Furthermore, PGE_2 has been indicated to hyperpolarize GB neurons, thereby inhibiting neurogenic contractions of GB (199). Normally, ROS produced during oxidative metabolism is cleared by antioxidant mechanisms, yet oxygen-derived free radical production may exceed the capability of scavengers, resulting in ROS accumulation and pathogenic effects during inflammation. Furthermore, during inflammation, excessive production of NO through inducible NOS with concurrent ROS production increases H_2O_2 formation (200,201). Exogenous H_2O_2 causes GBSM contraction and impairs GB responses to agonists of membrane-dependent receptors, thus inducing GBSM impairment (201,202). Histamine is released from mast cells, which are abundant in the GB wall. In GSMCs, histamine performs diametrically opposed functions through H_1 and H_2 receptors. Activation of H_1 receptors depolarizes GSMCs, whereas activation of H_2 receptors causes hyperpolarization via K_{ATP} channels (63,203). However, the net effect of histamine in GB is normally contraction (204). Although the role of histamine in AC is not fully understood, it is possible that AC is associated with increased mast cell infiltration and degranulation. ETs are bioactive peptides produced by GB epithelial cells, which have a crucial role in the early inflammatory process of AC. GB tissue ET levels are elevated, which is accompanied by an increase in GB tone (205). This pathological change precedes any histological evidence of GB inflammation. ET likely exerts an autocrine/paracrine role in the human GB via ET-a and ET-b receptors of GBSM (206). Pretreatment of the GB with an ET antagonist abrogated the development of AC.

In addition, decreased GB motility in AC results from the effects of neutrophils on the development and function of the ICCs network via depression of SCF/c-Kit expression (207). Upon coculture with neutrophils *in vitro*, the intracellular calcium transient of ICCs was less sensitive to contraction agonists and inhibitors (208). A study of human GB strips from AC suggested that overexpression of B_1 receptors by GSMCs may contribute to the typical symptoms that underline biliary colic during the cholecystitis state (142).

8. Conclusions

In summary, regulation of the membrane potential is complex, as GSMCs are electrically coupled to ICCs and TCs. Activation of conductance in any STIN cell affects the excitability of the syncytium. Individual STIN cells express intrinsic electrophysiological mechanisms and a variety of receptors for neurotransmitters, hormones, paracrine substances and inflammatory mediators. Similar to other GI SMCs, GSMCs rely on the formation of cross-bridges between actin and myosin for the development of force to empty the GB. The onset of GSMC depolarization requires SWs generated and propagated by GB ICCs. TCs (also known as PDGFRα^+ cells) exert an inhibitory effect on the excitability of SMCs through SK3 channels in the GI tract. However, the specific role of TCs in GB has yet to be studied and is a potential topic for future electrophysiological studies of GB. Therefore, the integrated output of the STIN syncytium sets the transient excitability of GSMCs. The primary risk factor for benign GB disease is GB dysmotility. Loss and dysfunction of STIN cells have been observed in patients and animal models with cholelithiasis and cholecystitis, suggesting that impairment of the STIN syncytium may be a critical pathogenic factor in benign GB disease. However, to date, there remains a lack of breakthroughs in the study of STIN syncytium. Thus, further research to better understand the pharmacology and physiology of the STIN syncytium is required.

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Availability of data and materials

The raw single-cell RNA-sequencing data that were used to generate Fig. 2 may be obtained at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179524.

Authors' contributions

FD and QH drafted the manuscript; MJ, RG, LL and FC prepared the figures and tables; YW and ZC critically revised the manuscript; HH and GZ conceived the review. HH and GZ checked and confirmed the authenticity of the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

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