ORIGINAL ARTICLE

Eur. J. Anat. 24 (1): 17-29(2020)

Immunohistochemical characteristics of ganglionated plexuses in the human gallbladder

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SUMMARY

Indirect triple immunolabelling techniques were used to identify the presence of choline acetyltransferase (ChAT), neuronal nitric oxide synthase (nNOS), Dopamine beta-hydroxylase (DβH), neuromedin U-8 (NMU-8), and neuropeptide Y (NPY) in the ganglionated plexuses of the human gallbladder.

Of all the intrinsic cholinergic neurons examined, NMU-8-immunoreactive (-IR) neurons appeared to be the most populous, followed closely by neurons containing NPY-IR. nNOS-IR neurons were often observed to coexist with ChAT, NMU, and NPY. Occasionally, these nNOS positive neurons were seen triple labeled with ChAT, NMU-8, and NPY. Results also indicate that not all nNOS and NMU-8 -IR coexistent neurons express NPY immunopositivity. Our findings suggest that these intrinsic neurons may be categorized into a distinct population of neurons that express both inhibitory and excitatory capabilities.

Intrinsic cholinergic neurons that were ChAT-IR displayed a spectrum of immunopositivity. Interestingly, a small subpopulation of these neurons appeared to be extremely weak ChAT-IR or simply ChAT-immunonegative. These occasionally observed ChAT-immunonegative neurons at times expressed single immunopositivity for NMU-8 or nNOS, while more frequently, these ChAT-immunonegative neurons were found to be single immunopositive, or at times, double immunopositive for NMU-8-, NPY-, or nNOS-IR.

Dopamine beta-hydroxylase (DβH) antibodies were used to confirm the lack of intrinsic sympathetic innervation in the human gallbladder. As suspected, in all the sections examined, DβH-IR neurons were not detected. The only indication of DβH immunopositivity was noted among delicate fibers surrounding the neurons and blood vessels within the organ.

Key words: Immunohistochemistry – Triple-labelled – Ganglionated plexus – Human – Gallbladder

INTRODUCTION

The human gallbladder is a small, pear-shaped organ located on the inferior surface of IV and V liver segments. This organ is approximately 8 cm (7-10 cm) in length and 4 cm in diameter that is comprised of three easily distinguishable layers: mucosa, muscularis propria, and adventitia (Kerr, 1999; Jones and Young, 2019).

In healthy gallbladders, the luminal surface of the mucosa is lined with simple columnar epithelium composed of distinctly tall cells with an oval-shaped nucleus and possesses microvilli on its

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Submitted: 21 August 2019. *Accepted:* 13 Novenber, 2019.

apical domain. These epithelial cells are responsible for water absorption whereby concentrating bile, as well as producing and secreting bicarbonates and mucins, which protects the epithelium against bile acids. The epithelium is resting on a thin non-cellular connective tissue layer known as the basement membrane. The basal layer of the basement membrane, also known as the reticular lamina, is attached to the lamina propria (Kerr, 1999; Housset et al., 2016).

The lamina propria is composed of loose areolar connective tissue with vascular plexus. It is interesting to note that there are no submucosa or muscularis mucosae within the inner layer of this organ. As a whole, the mucosa is highly folded, forming the rugae, which allows the organ to stretch when filled with bile. Numerous deep epithelial invaginations can be seen in sections that are referred to as crypts or Rokitansky-Aschoff sinuses (Kerr, 1999; Housset et al., 2016). At the base of the mucosal folds, occasional ganglionated plexuses are seen intermingling with the connective tissues.

The middle layer of the gallbladder is composed of an irregularly arranged layer of smooth muscle called muscularis propria. The contraction of this muscular layer is responsible for forcing the stored bile into the cystic duct. Randomly distributed and occasionally seen are ganglionated plexuses intermixed among the smooth musculature of the muscularis propria. Surrounding the muscularis propria, in areas that are connected to the liver, this muscular layer is connected to collagen and elastin-rich fibro-elastic connective tissue called adventitia. In areas that are not attached to the liver, an outer layer of the mesothelium (a layer of simple squamous epithelium) and loose areolar connective tissue form an outer covering called the serosa (Kerr, 1999).

The gallbladder, along with the cystic duct, is innervated by three routes. The right vagus nerve (a hepatic division of the vagus nerves), joining the anterior hepatic plexus in the hepatoduodenal ligament, is responsible for providing cholinergic innervation. The posterior hepatic plexus arising from the celiac branches of the posterior vagal trunk and the celiac plexus is responsible for adrenergic innervation of the organ. Sensory information from the organ is conveyed by the right phrenic nerve (Yi et al., 2007; Jones and Young, 2019).

The gallbladder is responsible for storing and concentrating bile between meals. Research has shown that the motor function of the gallbladder is regulated through G-protein-coupled bile acid receptor 1 (GPBAR1), also known as TGR5, found on L-cells and intestinal epithelium throughout the gastrointestinal tract (van Nierop et al., 2017). GPBAR1 receptors are used in the detection of bile acids, triggering the release of glucagon-like peptide-1 and -2 (GLP-1, -2) secretion from intestinal L-cells, and stimulate the filling of the gallbladder with bile (Li et al., 2011; Duboc et al., 2014; Yusta et al., 2017; Shapiro et al., 2018). Fibroblast growth factor 15/19 (FGF15/19), an intestinal hormone, has been found to stimulate gallbladder filling and act on hepatocytes to suppress gluconeogenesis, bile acid synthesis, and stimulate glycogen and protein synthesis (Kliewer and Mangelsdorf, 2016). Ileal enterocytes release FGF15/19 through the interaction of bile acids with farnesoid X receptor (FXR).

Additionally, cholecystokinin (CCK), a polypeptide hormone of neural and gastrointestinal origin secreted by enteroendocrine cells (I-cells) concentrated in the proximal regions of the duodenum, as well as the neurons in the walls of the intestines and the cerebral cortex, plays an important role in the motility of the gallbladder. It has been reported that ingested fats and proteins in the lumen of the duodenum trigger the increase in blood CCK concentration. It has been reported that gallbladder neurons lack CCK receptors; therefore, CCK acts on presynaptic nerves, which in turn trigger the release of acetylcholine. Increase acetylcholine release will prompt the contraction of the gallbladder to deliver bile, as well as elicit the release of pancreatic enzymes into the duodenum (Takahashi et al., 1991; Mawe, 1991; Otsuki, 2000; Rehfeld, 2017).

Although the fundamentals of gallbladder function have been accepted for decades, the hormonal and neuronal regulations of this organ have yet been fully elucidated. Since disturbances of the motility of the gallbladder and the sphincter of Oddi may contribute to gallstone disease, it is necessary to further our understanding of the manner by which the motility of this organ is controlled (Reshetnyak, 2012).

Previous studies have demonstrated the distribution patterns of neurotransmitters in nerve fibers and intrinsic neurons by using antibodies targeting enzymes involved in their formation, as well as direct antibody targeting of neuropeptides within the mammalian gallbladder (Lillemoe et al., 1988; Sand et al., 1993; Mawe et al., 1997; Mawe, 1998). However, only a few researchers attempted to describe the neurotransmitter (targeting the enzymes involved neurotransmitters formation) and neuropeptide innervation patterns of nerve fibers and intrinsic neurons in human tissue (De Giorgio et al., 1995; Gonda et al., 1995a; El-Salhy et al., 1996; Uemura et al., 1997; Meedeniya et al., 2001; Todorovic et al., 2003), while no triple labeling immunohistochemical investigations have ever been performed on the human gallbladder. In the present study, triple-labeling immunohistochemistry was used to examine the coexistence of the choline acetyltransferase (ChAT), neuronal nitric oxide synthase (nNOS), neuromedin U-8 (NMU-8), and Neuropeptide Y (NPY) in ganglionated plexus of the adult human gallbladder.

MATERIALS AND METHODS

Eleven human gallbladder tissue samples were obtained through contractual agreement and partnership with Pikeville Medical Center (PMC). These gallbladder samples were removed using laparoscopic cholecystectomy from patients suffering from cholelithiasis with biliary colic as sequelae of their conditions.

We included eleven cases of cholelithiasis (calculous gallbladder) in this study. On reviewing the reported cases, several comments can be made regarding clinical presentation and laboratory and imaging findings. Obesity was the most likely underlying disease, with 3 out of 11 (27.27%) had bariatric surgeries. Of the 11 cases enrolled in the study, gallstones were found surgically in only six gallbladders. Of these cases, 2 out of 6 (33%) were cholesterol stones, while the majority, 4 out of 6 (66%), were bile/bilirubin stones. All our patients were females and presented with chronic biliary colic (right upper quadrant abdominal pain and nausea exacerbated by fatty food). None of our patients had acute cholecystitis or pancreatitis. Most patients (10/11; 90.9%) had normal white blood cell counts. All patients had normal kidney function, platelet counts, thyroid function tests, lipid profile, and liver enzymes. All patients responded to surgical management, with no postsurgical complications and with remarkable clinical abdominal relief (Table 1).

Tissues were obtained with the written consent of the patient. Specimens collected were preserved using Zamboni's fixative solution. Samples were washed with phosphate-buffered saline (PBS) three times in ten-minute intervals using the New Brunswick Scientific Excella E24 Incubator Shaker. After washing, tissues were embedded onto the cryostat embedding mount using Sakura Tissue-Tek® OCT Compound and Bright Cryospray 134A and flash frozen. Embedded tissues were then cut into 10 µm thick sections using the Bright OTF 5000 Cryostat and applied to gel-coated microscope slides. One from every ten prepared slides was selected for Masson's trichrome staining. Once stained, the slides were dried, cleared, and mounted using Permount mounting medium. Remaining slides were stored at -80o C in a Helmer Scientific ultra-low temperature freezer.

Morphology

General histological examination of the eleven tissue samples reveals histopathological changes in the gallbladder mucosa due to cholelithiasis. In all the samples examined, the simple columnar cells of the epithelium have flattened to low columnar or high cuboidal shaped cells. Many of these epithelial cells appear to possess vacuolated cytoplasm. The epithelium appears to be discontinuous and disrupted, while the apical microvilli, which are prevalent in healthy tissues, were not readily seen or simply missing in the tissues examined.

Immunohistochemistry

Each trichrome-stained slide was examined to reveal the location of ganglionated plexus with in-

Table 1. Patient's characteristics

Patients' characteristics	Average findings
Age (years)	37.8 (23-59)
Sex	All females
White blood cell count (NR: 3-11.3 k/µl)	7.80 (4.4-13.2)
Hemoglobin (NR: 10-16 g/dL)	13.41 (11.8-14.7)
Platelets (NR: 122-454 k/µl)	256 (228-274)
Alanine aminotransferase (ALT) (NR: 12-78 U/L)	28.66 (16-62)
Aspartate aminotransferase (AST) (NR: 15-37 U/L)	26.33 (11-93)
Total bilirubin (NR: 0.0-1.0 mg/dL)	0.63 (0.3-1.1)
Alkaline phosphatase (NR: 45-117 U/L)	78.16 (60-104)
Creatinine (NR: 0.6-1.3 mg/dL)	0.68 (0.5-0.8)
Albumin (NR: 3.4-5.0 g/dL)	3.68 (3.4-3.7)
Glucose (NR: 70-110 mg/dL)	78.66 (63-87)
Total cholesterol (NR: <200 mg/dL)	150.33 (118-160)
High-density lipoprotein (HDL) (NR: 40-59 mg/dL)	51 (42-55)
Low-density lipoprotein (LDL) (NR: <100 mg/dL)	78.93 (46-99)
Triglyceride (NR: <150 mg/dL)	99.66 (81-126)
Thyroid-stimulating hormone (TSH) (NR: 0.4-4.0 mIU/L)	2.15 (1.39-3.3)
Abdominal ultrasound	Multiple gallbladder stones

NR: normal range

Table 2. Primary antibodies used in this experiment

Antigen	Host Species	Antibody Type	Dilution	Supplier
ChAT	Goat	Polyclonal	1:200	Thermo Fisher
DβH	Goat	Polyclonal	1:200	Thermo Fisher
NMU-8	Rabbit	Polyclonal	1:200	Thermo Fisher
NPY	Rabbit	Polyclonal	1:200	Thermo Fisher
NPY	Sheep	Polyclonal	1:500	Millipore
nNOS	Mouse	Monoclonal	1:400	Thermo Fisher

tramural nerve cells. Once suitable sections were found, slides were then selected for triple labeling. Slides were washed for 10 minutes in blocking buffer (.05% Sodium Azide, 1.5% goat serum, 0.1% Triton, and 98.35% PBS). A barrier was drawn around tissue on slides with the Vector Laboratories® ImmEDGE™ Hydrophobic Barrier Pen to prevent antibodies from displacing during incubation.

Neuromedin U-8 (NMU-8) or neuropeptide Y (NPY) polyclonal antibody (rabbit; 1:200; Thermo Fisher Scientific) was applied to the slide and left to incubate overnight. All incubations were performed in a dark incubation slide box stored at 4o C in a Fisher Scientific general purpose refrigerator. After incubation, slides were washed three times in ten-minute intervals with PBS using the Excella E24 incubator shaker. The secondary antibody, Biotinylated Anti-Rabbit IgG (H+L) (1:200; Vector Laboratories), was applied and left for four hours. Slides were again washed three times in ten-minute intervals with PBS. Fluorescein Avidin DCS (Cell Sorter Grade) (1:300; Vector Laboratories) was applied and left for three hours. The combination of the Anti-Rabbit IgG and the Fluorescein Avidin DCS were used to visualize the NMU-8 or NPY polyclonal antibodies. Slides were again washed three times in ten-minute intervals with PBS before mounted with a solution of 50/50 PBS and glycerol.

Neuronal Nitric Oxide Synthase (nNOS) monoclonal antibody (mouse; 1:400, Thermo Fisher Scientific) was administered to slides and left overnight for incubation. All incubations were performed in a dark incubation slide box stored at 4o C in a Fisher Scientific General Purpose Refrigerator. Slides were again washed three times in ten-minute intervals with PBS. To visualize the nNOS primary antibody, Donkey Anti-Mouse IgG Alexa Fluor 594 (1:200; Abcam) was applied and left to incubate for six hours. Slides were again washed three times in ten-minute intervals with PBS before mounted with a solution of 50/50 PBS and glycerol.

Choline Acyltransferase (ChAT), dopamine beta-hydroxylase (DβH) polyclonal antibody (goat; 1:200; Thermo Fisher Scientific), or NPY polyclonal antibody (sheep; 1:500; Millipore) was applied to slides and left overnight to incubate. All incubations were performed in a dark incubation slide box stored at 4o C in a Fisher Scientific General Purpose Refrigerator. Slides were again washed three times in ten-minute intervals with PBS. To visualize the ChAT polyclonal antibody, slides were incubated with Donkey Anti-Goat IgG H&L Alexa Fluor 405 (1:200; Abcam) or Donkey Anti-Sheep IgG H&L Alexa Fluor 405 (1:200; Abcam) for six hours. Slides were washed a final three times in ten-minute intervals with PBS before mounted with a solution of 50/50 PBS and glycerol (Table 2).

Analysis

Slides were viewed under an Olympus FluoViewFV1000 confocal microscope to determine the colocalization of the neurotransmitters (enzymes involved in neurotransmitter formation) and neuropeptides. All photomicrographs were taken at 400x magnification with 2x zoom.

RESULTS

Human gallbladder ganglionated plexuses observed within the scope of our experiment are composed of an array of small neurons that can be organized into two populations. One population is located in the lamina propria, while the other is found within or near the muscularis propria. Triple-labeling techniques were used to demonstrate Immunoreactivity to enzymes involved in neurotransmitter formation, and neuropeptides are found in neurons of ganglionated plexuses of the human gallbladder. Five sets of triple-labeling were used on the sectioned tissues: ① ChAT, nNOS, and NMU-8; ② ChAT, nNOS, and NPY; ③, NPY (sheep), nNOS, and NMU-8; ④ DβH, nNOS, and NMU-8; ⑤ DβH, nNOS, and NPY (Table 3).

Nearly all neurons found in both the lamina pro-

Table 3. Triple-label combinations used in this study

Staining	Triple-labeled Combinations
1	ChAT (goat) - nNOS (mouse) – NMU-8 (rabbit)
2	ChAT (goat) – nNOS (mouse) – NPY (rabbit)
3	NPY (sheep)– nNOS (mouse) – NMU-8 (rabbit)
4	DβH (goat) – nNOS (mouse) – NMU-8 (rabbit)
5	DβH (goat) – nNOS (mouse) – NPY (rabbit)

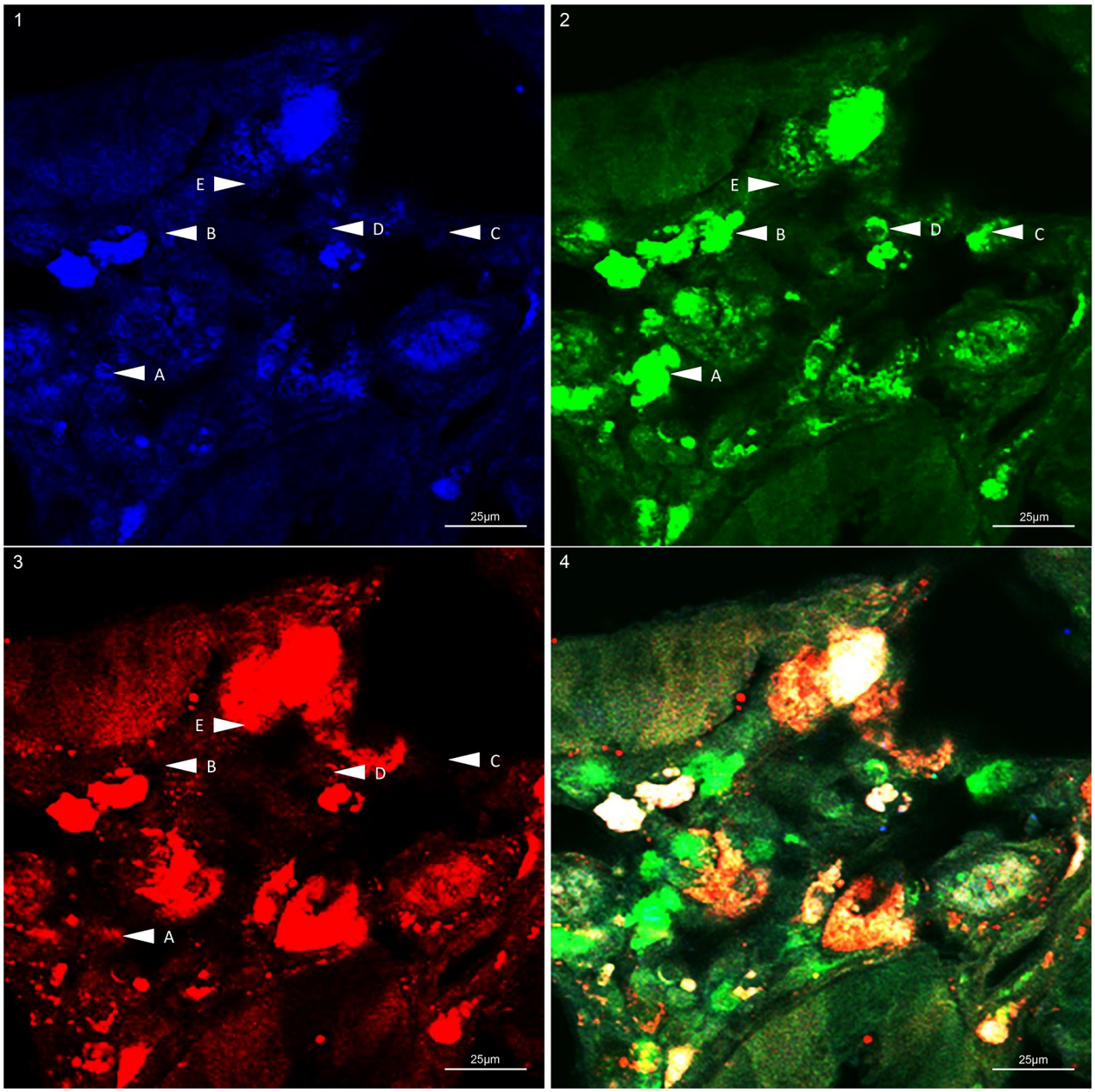

Fig 1. Triple labeled ganglion found in the muscularis propria of the human gallbladder demonstrating immunopositivity for ChAT, NMU-8, and nNOS. Although most of the neurons stained positive for all three antibodies, a small subpopulation of ChAT-immunonegative neurons exist. For example, arrow **A** indicates a cluster of NMU-8-IR neurons, with one neuron show weak immunopositivity for ChAT (neuron located at the top of the cluster) and nNOS (neuron located slightly below the ChAT-IR neuron). Arrows **B** and **C** demonstrate a cluster of NMU-8-IR neurons only. Arrow **D** indicates a neuron immunonegative for ChAT but immunopositive for NMU-8. This neuron appears to be surrounded by nNOS-IR axonal terminals. Arrow **E** indicates nNOS-IR neurons only. **1**. ChAT, **2**. NMU-8, **3**. nNOS, **4**. Triple labeled.

pria and the muscularis propria in all tissues examined were immunopositive for ChAT, although the immunopositivity varied in intensely from weakly to extremely positive. Of all the slides examined, no neurons immunopositive for DβH (presumptive adrenergic) were seen. The only indication of DβH positivity was detected as delicate fibers in the smooth musculature, within the ganglion, axonal terminals, and/or surrounding the blood vessels. In all the DβH triple labeled slides examined, only on occasion were these presumptive adrenergic fibers also demonstrate immunopositivity for nNOS.

The majority of the neurons in all the sections examined were NMU-8-IR. These NMU-8-IR neurons were frequently colocalized with ChAT, which indicates the importance of this excitatory neuropeptide in the innervation of this organ. Nonetheless, a small subpopulation of NMU-8-IR neuronal cell bodies was seen independent of the cholinergic marker (Figs. 1-4). NPY-IR neurons were also commonly seen coexisting with ChAT, although the frequency of detection was not as often seen with those presumptive cholinergic neurons co-

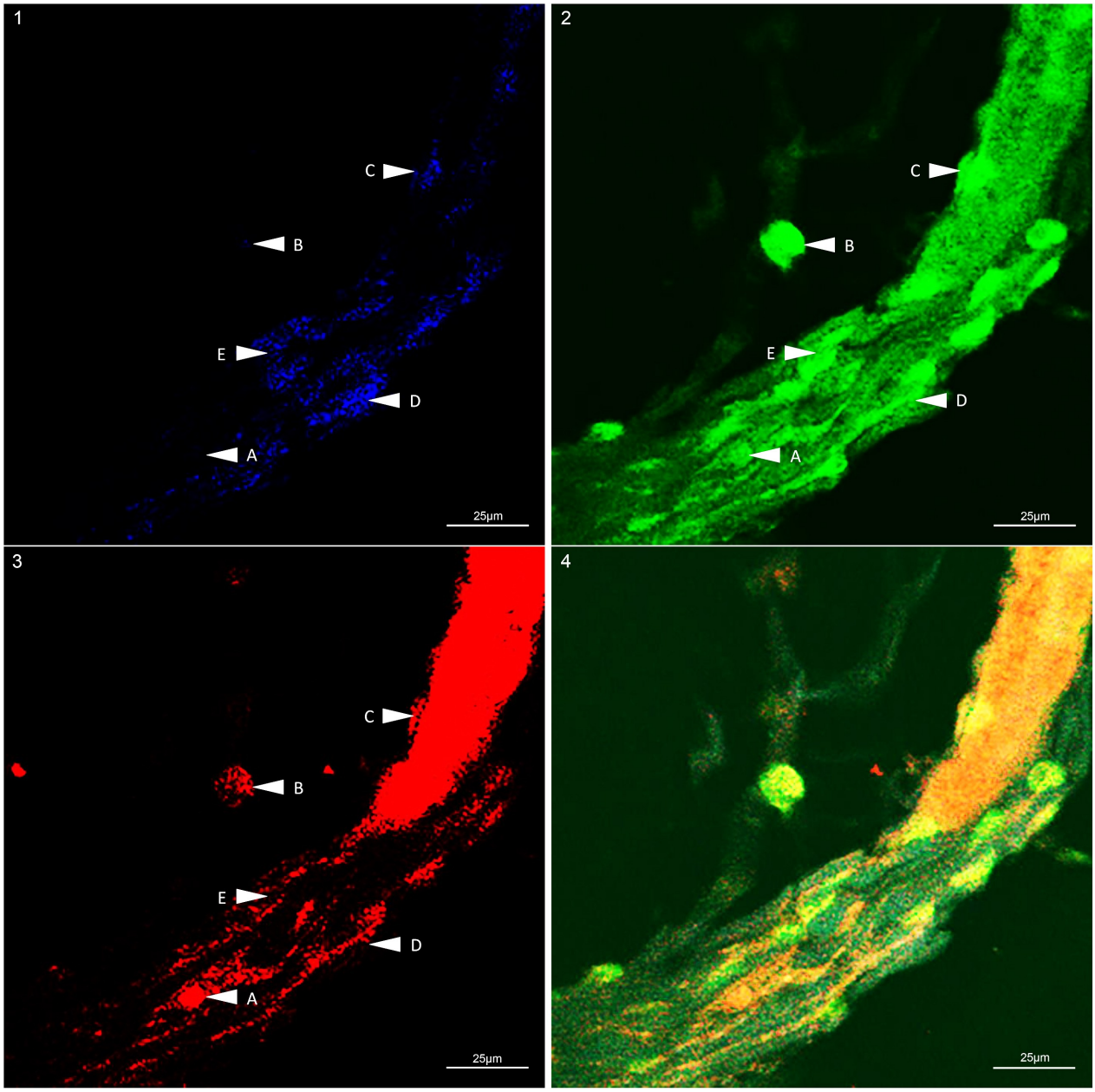

Fig 2. Triple labeled ganglion found in the lamina propria of the human gallbladder demonstrating immunopositivity for ChAT, NMU-8, and nNOS. Please note that most of the neurons are NMU-8-IR only. Nonetheless, numerous ChAT-IR and nNOS-IR nerve fibers and axonal terminals surrounding the neurons are seen. Arrow **A** shows a ChAT-immunonegative but NMU-8 and nNOS-IR neuron. Arrow **B** shows a ChAT-immunonegative but NMU-8 and nNOS-IR neuron. Arrow **C** demonstrates a bundle of intensely positive nNOS-IR fibers surrounding an NMU-8-IR and nNOS-IR neuron. Please note that some axonal terminals are seen that are ChAT-IR. Arrow **D** show triple labeled ChAT-IR, NMU-8-IR, and nNOS-IR nerve fibers. Arrow **E** demonstrates two NMU-8-IR neurons surrounded by nNOS-IR axonal terminals, where some of these nerve endings also show coexisting ChAT-IR. **1**. ChAT, **2**. NMU-8, **3**. nNOS, **4**. Triple labeled.

existing with NMU-8. It is interesting to note that a population of NPY-IR neurons was found to be immunonegative for ChAT (Fig. 5).

The existence of colocalized ChAT with NMU-8, or ChAT with NPY, and the small subpopulation of ChAT independent, NMU-8, or NPY neurons prompted additional staining using sheep anti-NPY antibodies to be employed. This additional step confirmed that in the neurons examined, NPY nearly always coexisted with NMU-8, although small populations of NPY-IR only neurons were infrequently seen (Fig. 6).

Immunopositive nNOS neurons, on the other hand, were less frequently observed. When they are identified, these nNOS-IR neurons often co-existed with ChAT, NPY, and NMU-8. It is interesting to note that the existence of inhibitory NPY and nNOS coexisting with excitatory ChAT and NMU-8 demonstrates that these ganglionated plexuses examined express a distinct crossover po-

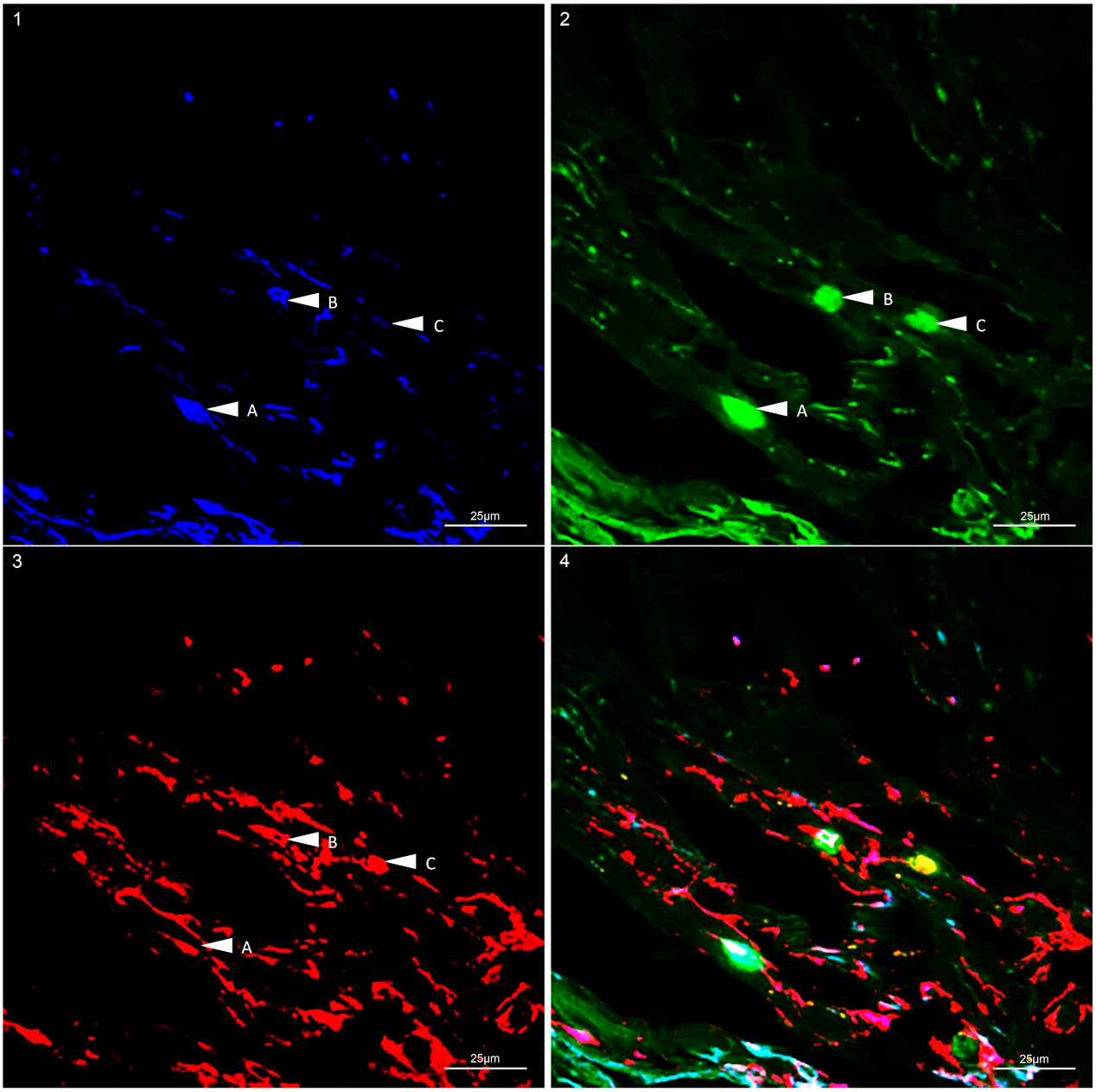

Fig 3. Triple labeled section of the muscularis propria of the human gallbladder, demonstrating immunopositive ganglion cells and numerous triple labeled nerve fibers. Please note that some of the intramuscular fibers stained positive for all three antibodies, while some showed the coexistence of ChAT and NMU-8. Interestingly, as shown in pictures 3 and 4, many of the nerve fibers contain only nNOS-IR. Arrow **A** and **B** show a ChAT-IR, nNOS-IR, and NMU-8-IR triple labeled neuron. Arrow **C** indicates the appearance of ChAT-immunonegative but nNOS and NMU-8-IR neurons. **1**. ChAT, **2**. NMU-8, **3**. nNOS, **4**. Triple labeled.

pulation of neurons that express both inhibitory and excitatory capabilities (Table 4). On rare occasions, neurons immunopositive for only nNOS, or coexisting nNOS/NMU-8, or nNOS/NPY were observed (Fig. 6).

Numerous nerve fibers were seen coursing among the neurons within ganglia found in both lamina propria and muscularis propria. Some of these nerve fibers are ChAT-, nNOS-, and NMU-8-IR, while others possess only ChAT-/nNOS-IR or nNOS-/NMU-8-IR. Interestingly, the majority of these nerve fibers were immunopositive for nNOS only (Fig. 2). Numerous axonal terminals surrounding the neurons were observed. Frequently, DβH/nNOS-IR axonal terminals were seen surrounding presumptive cholinergic neurons. Occasionally, some of these axonal terminals were shown to display nNOS-IR only. Only on occasion were double-positive ChAT-/nNOS-IR, or triple positive ChAT-/nNOS-/NMU-8-IR axonal terminals observed (Fig. 2).

DISCUSSION

The distribution of the intramural ganglionated plexus of the human gallbladder is composed of an

Table 4. Estimated distribution of neuropeptides and neurotransmitter synthesizing enzymes in the ganglionated plexus of the human gallbladder. Please note: the estimate for individual existence or dual existence (double-labeled) of neuropeptides and neurotransmitter synthesizing enzyme is part of the triple-labeled experiments. No single- or double-labeling experiments were performed.

Antigen	Lamina Propria	Muscularis propria
ChAT only	-	-
DβH only	-	-
NPY only	+	+
nNOS only	+	+
NMU-8 only	+	+
ChAT/nNOS[1]	-	-
ChAT/NMU-8[2]	-	-
ChAT/nNOS/NMU-8	+++	+++
ChAT/nNOS/NPY	+++	+++
DβH/NPY	-	-
DβH/nNOS	-	-
DβH/nNOS/NMU-8	-	-
DβH/nNOS/NPY	-	-
NPY/NMU-8[3]	+	+
nNOS/NMU-8[3]	+	+
nNOS/NPY[3]	+	+

+++ High, ++ Moderate, + Sparse, - Not observed, [1]NMU or NPY negative, [2]nNOS negative, [3]ChAT negative

array of small neurons distributed in the lamina propria and the muscularis propria. Although existing in two populations, the isolation of the individual ganglionated plexus appeared to be relatively random. Previous research has shown that autonomic nerves modulate the actions of these neurons (e.g., extrinsic sympathetic, parasympathetic), extrinsic sensory, duodenal neural inputs, bile acid influencing GPBAR1 and subsequent secretion of GLP-1 and -2 by intestinal L-cells (Li et al., 2011; Duboc et al., 2014; Yusta et al., 2017; Shapiro et al., 2018)., and prompted by circulating hormones such as CCK, secretin, gastrin, FGF15/19, and pancreatic polypeptide (Mawe, 1998; Otsuki, 2000; Mawe, 2000; Rehfeld, 2017; Kliewer and Mangelsdorf, 2016).

The vast majority of the neurons that are responsible for the innervation of the gallbladder were observed to be ChAT-IR, although a spectrum of immunopositivity ranging from strong to weak was seen. This observation is consistent with the findings that described that all gallbladder neurons are cholinergic (Mawe, 1998; Talmage et al., 1996; Gonda et al., 1995b) and that the contraction of this organ involved cholinergic nerves (Yamasato and Nakayama, 1990) acting on muscarinic receptors (Uemura et al., 1997). In addition to ChAT-IR, additional research in mammalian species, including humans, has shown that these presumptive cholinergic neurons coexist with various neurochemicals. It is believed that the neurochemical contents of gallbladder neurons are to provide fine control to the organ's motility, as well as regulating epithelial function (Peterson et al., 1993, De Giorgio et al., 1995, Talmage et al., 1996, Uemura et al., 1997). For example, described in a review article by Balemba et al. (2004), these cholinergic neurons can express several neuronal active compounds such as substance P (SP), galanin (GAL), nitric oxide (NOS), and vasoactive intestinal peptide (VIP) (Balemba et al., 2004). Although SP, GAL, and VIP were not examined in this study, the existence of nNOS in the intramural ganglia is in agreement with their findings. Uemura et al. (1997) indicated that NOS-IR nerve cell bodies comprised approximately 13% of the nerve cells found in the fibromuscular layer (muscularis propria), and approximately 30% of the nerve cells in the subepithelial layers (lamina propria) (Uemura et al., 1997). Muscularis propria NOS-IR intramural neurons are believed to play an inhibitory role in the musculature of the gallbladder, which, in turn, affects the filling process of the gallbladder. It has been reported that the filling process of the gallbladder involves receptive relaxation, a neurologically controlled process, which increases the volume without causing a rise in intraluminal pressure (Cole et al., 1987; Mourelle et al., 1993). On the other hand, the nNOS-IR subepithelial neurons positive may be involved in the regulation of epithelial secretions (Uemura et al., 1997; Parkman et al., 1997; Greaves et al., 1998), and/or epithelial absorption of water and electrolytes that concentrates bile containing hydrophobic bile salts (Cole et al., 1987; Behar, 2013). In the present study, most of the nNOS-IR neurons also demonstrated immunopositivity for NMU-8 and/or NPY.

Originally isolated from porcine spinal cord, the bioactive peptide neuromedin U (NmU) 25 and 8 (shares the C-terminal sequence of NmU-25) has been characterized by its potent contractile activity in smooth musculature of various mammalian species (Minamino et al., 1985; O'Harte et al., 1991; Prendergast et al., 2006), including in the human gallbladder (Jones et al., 2006). Additionally, this neuropeptide has been shown to possess the ability to serve as an endogenous peptide of the central nervous system in playing a role in the regulation of feeding and energy homeostasis (Ingallinella et al., 2012). NPY, on the other hand, has been reported in the neurons of the human and porcine gallbladder (De Giorgio et al., 1997; Sand et al., 1993). According to Uemura et al. (1997), NPY serves as an inhibitory neuropeptide and is involved in gallbladder filling (Uemura et al., 1997).

In the present study, the localization of nNOS with ChAT and NMU-8, or NPY, demonstrates the

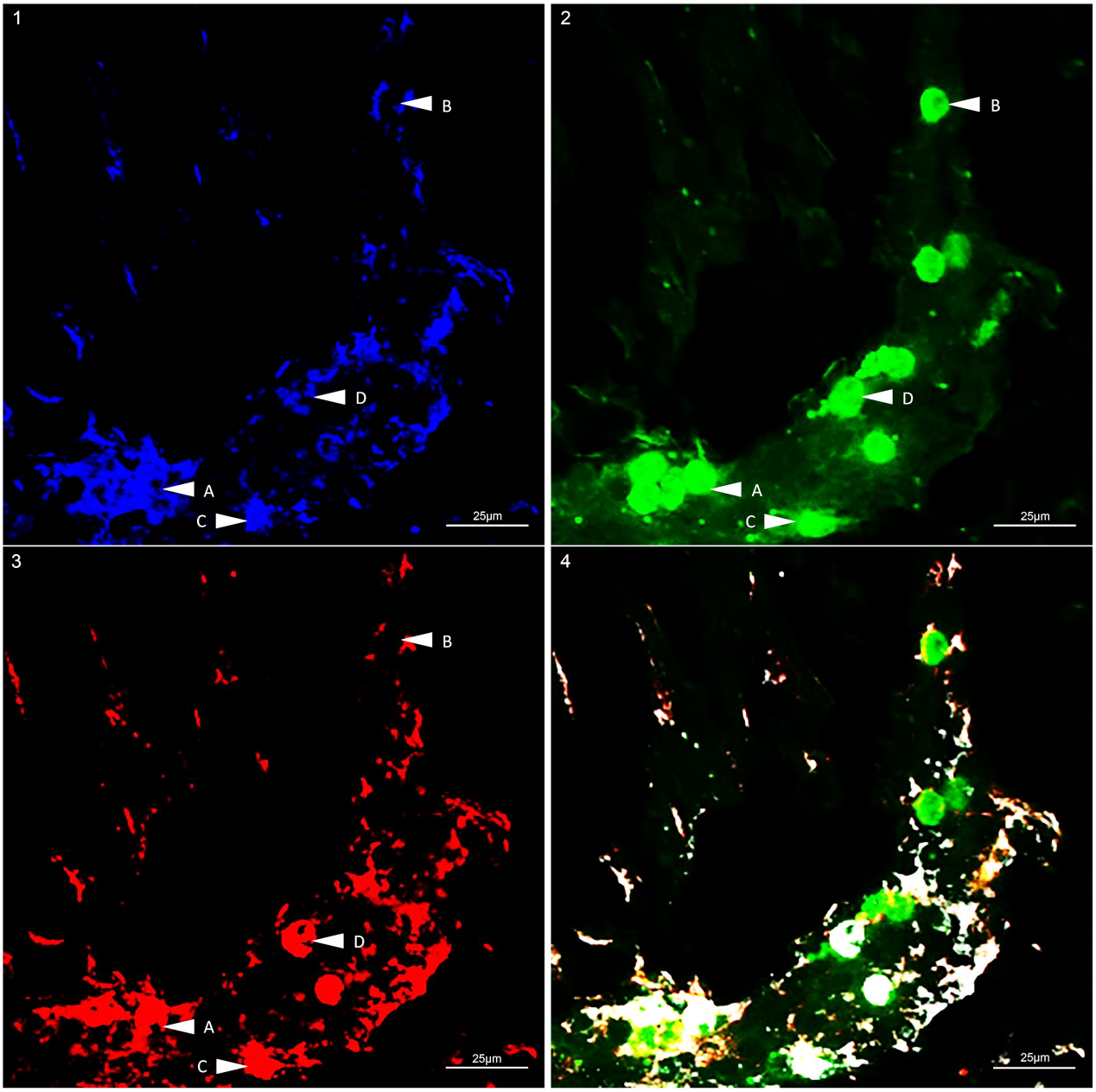

Fig 4. Triple labeled section of the lamina propria of the human gallbladder, demonstrating immunopositive ganglion cells and numerous triple labeled nerve fibers. Please note that some of the nerve fibers stained positive for all three antibodies, while some showed the coexistence of ChAT and NMU-8. Interestingly, as shown in pictures 3 and 4, many of the nerve fibers contain only nNOS-IR. Arrow **A** shows a cluster of neurons where some are triple labeled ChAT, nNOS, and NMU-8, while others show immunopositivity for ChAT and NMU-8 only. Please note that there are numerous ChAT and nNOS-IR axonal terminals that are seen surrounding the neurons. Arrow **B** shows a single positive NMU-8-IR neuron surrounded by ChAT and nNOS-IR axonal terminals. Arrow **C** and **D** indicate a triple labeled neuron. **1**. ChAT, **2**. NMU-8, **3**. nNOS, **4**. Triple labeled.

existence of the presumptive excitatory neurotransmitter forming enzyme ChAT, and neuropeptide NMU-8, with presumptive inhibitory neurotransmitter forming enzyme nNOS and neuropeptide NPY. This observation seems to be consistent with studies that demonstrated the presence of both NOS and ChAT in the guinea pig gallbladder ganglia (Talmage et al., 1996), and Australian Brush-tailed possum (Meedeniya et al., 2001). Additionally, various authors also indicated that in the human and guinea pig, vasoactive intestinal polypeptide (VIP) is colocalized with nNOS. Although in our present research VIP antiserum was not employed, it would be safe to assume that many of the nNOS-IR intramural neurons may also contain VIP (Gonda et al., 1995b; Talmage et al., 1996; Uemura et al., 1997; Meedeniya et al., 2001).

At first glance, the appearance of a small population of ChAT-immunonegative neurons appears to challenge the classical concept of the autonomic innervation. These ChAT-negative cells are often NMU-8-IR, and at times coexist with NPY and nNOS. However, upon closer examination of available data, the existence of these neurons may be

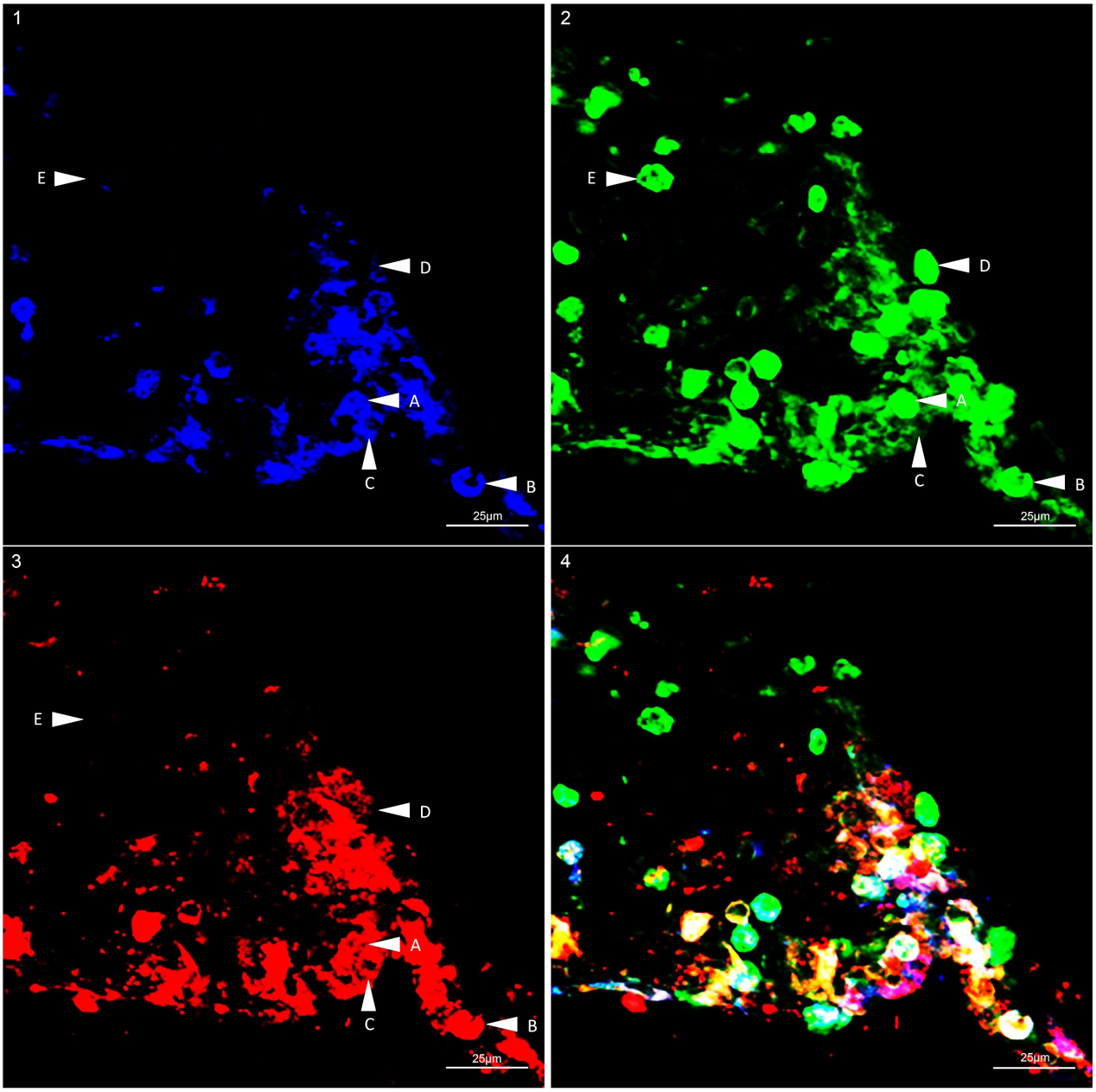

Fig 5. Triple labeled section of the lamina propria of the human gallbladder, demonstrating immunopositive ganglion cells and numerous triple labeled nerve fibers. Arrows **A** and **B** show a triple labeled ChAT-, nNOS-, and NPY-IR neuron. Arrow **C** demonstrates an NPY-immunonegative, but ChAT-/nNOS-IR neuron. Numerous neurons possessing only NPY-IR were seen as indicated by Arrows **D** and **E**. **1**. ChAT, **2**. NPY, **3**. nNOS, **4**. Triple labeled.

due to the concentration of ChAT enzymes within these neurons. According to a report by Meedeniya et al. (2001), the intensity of ChAT immunoreactivity varied among neurons, even within the same ganglion (Meedeniya et al., 2001). These researchers believe that, although all neurons within the gallbladder possess ChAT for acetylcholine synthesis, the use of this neurotransmitter may fluctuate from neuron to neuron. Nonetheless, this interesting observation will require further elucidation.

An interesting observation was made when numerous nNOS-IR axonal terminals were seen surrounding presumptive cholinergic neurons. In all the sections examined, occasional double-positive ChAT-/nNOS-IR or triple-positive ChAT-/nNOS-/NMU-8-IR axonal terminals were seen. Sporadically, some of these axonal terminals were shown to display nNOS-IR only. Most often, DβH/nNOS-IR axonal terminals were seen surrounding presumptive cholinergic neurons. This observation is consistent with previous reports that indicate that presynaptic sympathetic nerves terminate at the intramural ganglia and appear to contradict the effect of CCK. It is suggested that the norepinephrine secreted by these axonal terminals acts on the α2 adrenoreceptors and suppresses acetylcholine release and gallbladder contraction. Similarly, according to a report by Greaves et al. (1998), nitric oxide (NO) contradicts CCK induced contraction

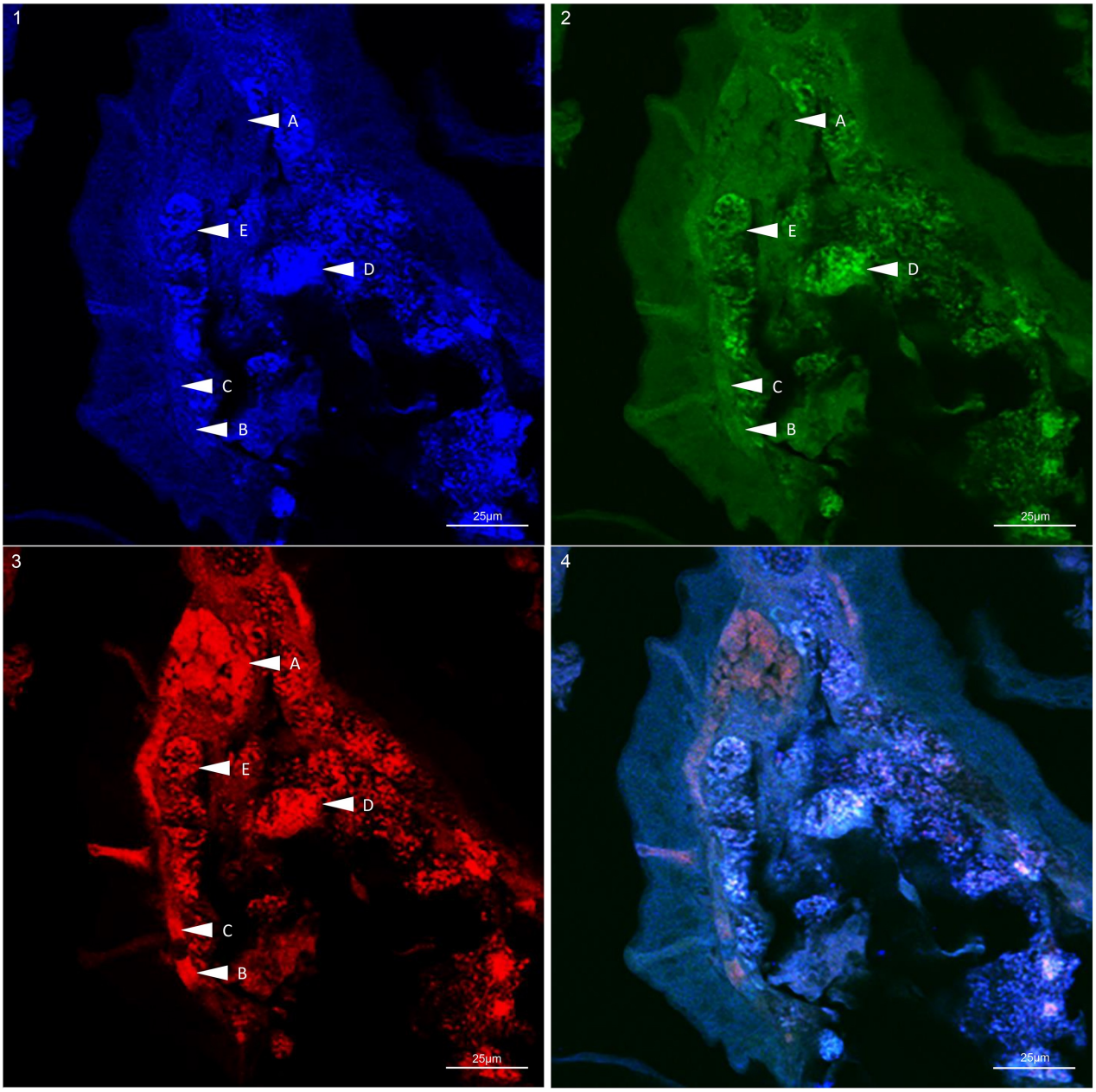

Fig 6. NPY, nNOS, and NMU-8 triple labeled section of the lamina propria, immediately beneath the simple columnar cells of the epithelium, of the human gallbladder, demonstrating numerous immunopositive ganglion cells. Arrow **A**, **B**, and **C** show a cluster of neurons that are immunonegative for NPY and NMU while exhibiting nNOS-IR only. Arrow **D** shows a triple-labeled ChAT-, nNOS-, and NPY-IR neuron. Arrow E shows a coexisting NPY- and nNOS-IR neuron immunonegative to NMU. **1**. NPY, **2**. NMU-8, **3**. nNOS, **4**. Triple labeled.

and triggers gallbladder relaxation (Greaves et al., 1998). This inhibitory effect of NO may be its direct action on cholinergic neurons in the intramural ganglia (Greaves et al., 1998), which, in turn, prohibit acetylcholine release (Shaffer, 2000).

In conclusion, the present study has shown that numerous immunohistochemically distinct populations of neurons exist within the ganglionated plexuses of the human gallbladder. These combinations indicate that numerous groups of neurons that possess both inhibitory and excitatory functions exist within the human gallbladder. The manner by which these neurons operate in the filling and emptying of the human gallbladder will require additional examinations in order to determine their precise functional role within this organ.

ACKNOWLEDGEMENTS

This study was supported by grants offered by Colonel Lee B. Ledford Scholarship from Appalachian College Association. The authors would like to express our most profound appreciation to Amari Eadie, Alyssa Cowger, Meera Singh, and Dr. Tim Whittier for their support and expertise. We would also like to extend our gratitude to Pikeville Medical Center and the University of Pikeville for

their inter-institutional cooperation, which allowed this collaboration to occur.

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