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Distribution of Butyrylcholinesterase in the mouse gastrointestinal tract: a morphological study

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1 RIASSUNTO

La Butirrilcolinesterasi (BChE) è un enzima idrolitico appartenente alla famiglia delle carbossilesterasi prodotto e secreto dal fegato. È presente in tutti i mammiferi e ricopre un ruolo molto importante nella neurotrasmissione grazie alla sua azione nei confronti del neurotrasmettitore acetilcolina (ACh). Diversamente dal ruolo ben definito dell'acetilcolinesterasi (AChE) nella regolazione del signalling colinergico, per molti anni non è stata attribuita una vera funzione fisiologica alla BChE. Nonostante alcune informazioni relative alla sua attività enzimatica siano già state scoperte, un ruolo fisiologico chiaro deve ancora essere attribuito a questo enzima. Studi recenti hanno accresciuto l'interesse nei suoi confronti delineando un coinvolgimento della BChE nella regolazione dell'appetito e nell'insorgenza dell'obesità, individuando tale enzima come possibile responsabile dell'idrolisi dell'ormone grelina. Dato l'interesse crescente per la BChE e il suo rapporto con aspetti metabolici nell'ambito della regolazione dell'appetito, in questo lavoro di tesi è stata realizzata una mappatura morfologica dettagliata della distribuzione di questo enzima nel tratto gastrointestinale di topo. Attraverso tecniche di immunoistochimica e studi di immunofluorescenza con doppie marcature abbiamo realizzato una mappatura dell'enzima dalla porzione più rostrale (ghiandole salivari ed esofago), fino alla più caudale (intestino tenue e colon). Per ogni organo del tratto gastrointestinale analizzato le reazioni di immunoperossidasi qui svolte mostrano la distribuzione della BChE, mentre le doppie marcature identificano attraverso microscopia a fluorescenza confocale gli esatti citotipi che esprimono l'enzima. Cellule positive per la BChE sono state identificate nel fegato, nella porzione duttale delle ghiandole salivari, nell'epitelio cheratinizzato dell'esofago, nella porzione ghiandolare della mucosa gastrica (cellule parietali), nelle cripte intestinali (cellule di Paneth), e nelle cellule a secrezione mucosa del colon e duodenali (ghiandole di Brunner). È interessante notare come la BChE sia presente vicino alle nicchie proliferative gastrointestinali. È stata riportata qui per la prima volta la presenza di BChE nei colangiociti epatici, nelle cellule di Paneth e nella porzione ghiandolare dello stomaco: risultati che possono suggerire un ruolo di controllo per la BChE nei confronti dell'omeostasi e della rigenerazione tissutale. Un altro possibile ruolo fisiologico per la BChE derivante dall' interpretazione dei nostri dati potrebbe essere la sua azione come agente detossificante nei confronti dei composti introdotti con la dieta. Inoltre, la correlazione spaziale tra le cellule che producono grelina e le cellule parietali positive alla BChE nella mucosa ossintica gastrica, potrebbe suggerire un ruolo idrolitico verso la grelina tramite un'azione paracrina, fin'ora mai proposto. Complessivamente, i dati morfologici proposti in questo lavoro di tesi possono essere visti come un

importante punto di partenza per progetti sperimentali e studi futuri volti a chiarire l'esatto ruolo della BChE nel tratto gastrointestinale.

1 ABSTRACT

The Butyrylcholinesterase (BChE) is an hydrolytic enzyme produced and released by liver belonging to the carboxylesterases superfamily. It is present in all mammals and plays an important role in neurotransmission due to its action towards the neurotransmitter Acethylcholine (Ach). In contrast to the long-established and well-defined role of Acethylcholinesterase (AChE) in regulating cholinergic signaling, a true physiological function for BChE remained elusive over many decades. Despite BChE enzymatic activity have already been elucidated, a proper physiological role for that enzyme has yet to be defined. Recent studies rise the interest towards BChE proposing a new challenging and interesting role for that enzyme linked to obesity and appetite regulation: the hydrolysis of the neuropeptide gut hormone, ghrelin. Due to the growing interest for BChE and its role in the field of feeding and appetite regulation, in this thesis a detailed morphological study of the distribution of BChE along the mouse gastrointestinal tract has been performed. Through immunohistochemistry and double labelling immunofluorescence studies, a map of the distribution of the enzyme has been proposed here from the more rostral portion (salivary glands and esophagus) to the more caudal one (large intestine and colon). For each organ of the gastrointestinal tract analyzed, the immunoperoxidase stainings described the distribution of BChE, while the double labelling studies identified the exact cytotype expressing this enzyme. BChE-positive cells were found in the liver, in the ductal portion of salivary glands, in the keratinized layer of the squamous epithelium of the esophagus, in the gastric mucosa (parietal cells), in the intestinal crypts (Paneth cells) and in mucus secreting cells of the duodenal Brunner glands and of the large intestine. Interestingly, the presence of BChE was frequently associated withto the distribution of gastro-intestinal proliferative niches. The presence of BChE in hepatic cholangiocytes, in intestinal Paneth cells and in the glandular portion of the stomach has been reported here for the first time: these results may suggest a role for BChE in the homeostatic control of tissues regeneration. Another possible physiological function for BChE coming from the interpretation of our results could be its action as a detoxifying agent towards ingested compounds. Moreover, the spatial correlation between ghrelin producing cells and BChE-positive parietal cells in the gastric oxyntic mucosa could suggest a BChE hydrolytic function towards ghrelin through a paracrine action.

Collectively, the morphological results coming from this thesis work can be proposed as the onset for further experimental projects and studies aiming to clarify the role of BChE in the gastrointestinal tract.

2.INTRODUCTION

2.1 The cholinergic system

The term cholinergic system is commonly used for the well-known neurotransmitter Acetylcholine (ACh) and the system of synthesizing enzymes, transporters, receptors and enzymes for degradation (Beckmann and Lips, 2013). ACh was the first substance proven to be a neurotransmitter (Loewi, 1921). The neuronal cells which compose the cholinergic system, during the propagation of a nerve impulse, are activated by or contain and release the neurotransmitter ACh which is involved in the transduction of action potentials and has been associated with numbers of cognitive functions including memory, selective attention, and emotional processing (Bertrand and Wallace, 2020). First identified by Dale (Broomfield et al., 1991) for its actions on heart tissue, ACh was later identified as a neurotransmitter molecule by Loewi (Loewi, 1921), who initially named it “Vagusstoff”, literally translated from German as "Vagus Substance", because it was released from the vagus nerve. Since then, the intricate processes of ACh synthesis and synaptic communication have been identified so that cholinergic synthesis and reuptake in neurons are now well understood. ACh is the major parasympathetic mediator and its synthesis requires choline, a natural amine found in the lipid bilayer of the cell membrane, and acetyl coenzyme A (acetyl-CoA), a thioester used in metabolic reactions and an acceptor and donor of acetyl groups. The reaction is catalyzed by choline acetyltransferase (ChAT), an enzyme found in cholinergic cells, aimed at producing AChE (Hebb, 1954; Hebb and Whittaker, 1958) and detectable in different neurons as well as in non-neuronal cells. Apart from central nervous system and the enteric nervous system (ENS), where ACh acts as neurotransmitter, ACh was also found in numerous non-neuronal mammalian cells (first reported in 1978) (Sastry and Sadavongvivad, 1978) such as human keratinocytes, airway cells and intestinal surface epithelial cells, where it is predicted to function as a local cell signaling molecule. ACh and associated metabolic enzymes have also been detected in more primitive life forms, such as plants, lichens, fungi, and even bacteria (Horiuchi et al., 2003) suggesting that it is a universal cell signaling molecule in multiple biological systems. In fact, ACh is not only considered a neurotransmitter, but is identified as an ancient molecule that can be released by and act on non-neuronal cells composing the non-neuronal cholinergic system (NNCS) together with ACh-synthesizing enzymes, transporters, receptors and degrading enzymes. Even before the nervous system was developed, the non-neuronal cholinergic system (NNCS) has already been described in non-neuronal cells like bacteria, algae and protozoa (Wessler et al., 1999).

Rediscovered in 1990s, the NNCS was started to be studied focusing on its distribution, functions, molecular components and its involvement in pathological conditions. So that it was found and described in many organ systems including skin keratinocytes, cardiovascular system, respiratory system, immune system, digestive system, urinary and reproductive system (Beckmann and Lips, 2013). In neurons and most non-neuronal cells, ACh is synthesized by the enzyme choline acetyltransferase (ChAT) from choline and acetyl coenzyme-A (Wessler et al., 2003; Wessler et al., 1999). It was shown that ACh can also be produced by the enzyme carnitine acetyltransferase (CarAT) in some non-neuronal cells, as skeletal muscle cells and the urothelium (Lips et al., 2007; Tucek, 1982). Once produced, the neurotransmitter is released from storing vesicles locally in the synaptic cleft (Beckmann and Lips, 2013; Wessler and Kirkpatrick, 2008). In cholinergic neurons, subsequent to its production and before being released, ACh is transported into synaptic cytoplasmic vesicles via the vesicular ACh transporter (VAChT), where it is stored until its release through exocytosis. After the release into the synaptic cleft, the neurotransmitter ACh exerts its effects by binding to two main types of receptors with two different structures and functional pathways: nicotinic receptors and muscarinic receptors (Bertrand and Wallace, 2020). They belong to two different membrane-bound receptor classes: G protein-coupled muscarinic ACh receptors (mAChRs), and ligand-gated nicotinic ACh receptors (nAChRs). The muscarinic receptor types are present both in the peripheral and central nervous system, the nicotinic types exert their functions in the peripheral and central nervous system, in the neurons from the parasympathetic ganglia, at the neuromuscular junction, as well as in non-neuronal cells (Bertrand and Wallace, 2020). The activation of one or another receptor type normally lasts less than a millisecond because a family of enzymes called cholinesterase rapidly breaks down ACh. The two products of the hydrolysis of the molecule, choline and acetic acid, undergoes a reuptake which occurs through a high-affinity choline transporters, and then choline is recycled for the synthesis of new ACh (Lockman and Allen, 2002). Hence, the role of cholinesterases, a superfamily which comprises Acetylcholinesterase and Butyrylcholinesterase, at the synaptic cleft results fundamental since allow cholinergic neurons to return to their resting state after activation.

2.1.1 The Cholinesterases

Cholinesterase is a family of carboxylesterase present in all mammals, which plays an important role in neurotransmission. Cholinesterases are specialized carboxylic ester hydrolases that break down esters of choline. In particular, they catalyze the hydrolysis of choline esters to produce an acid and an alcohol (figure1), and their most important and diffused substrate is ACh. These reactions are necessary to allow a cholinergic neuron to return to its resting state after activation, or to allow a muscle to relax after its contraction, or to end a hormone signaling after its activation, or to detox the organism from toxic compound exposures, depending on the diverse substrates on which they exert their action.

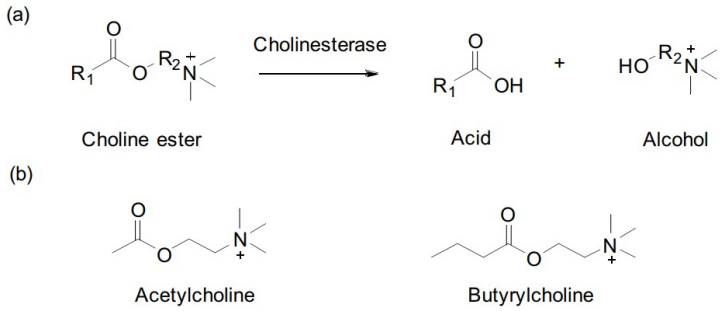

Fig. 1| *The hydrolysis of a choline ester into an acid and alcohol by cholinesterase*. (**b**) The molecular structure of ACh, the cholinergic neurotransmitter, and butyrylcholine, a synthetic choline ester tailored for Butyrylcholinesterase (Ha et al., 2020).

There are two existing types of cholinesterase, both widely studied: Acetylcholinesterase, AChE, also referred to as "true" or "specific" cholinesterase, and Butyrylcholinesterase (BChE or BuChE), discovered later and referred to as "serum cholinesterase", "pseudocholinesterase" or "non-specific cholinesterase". The synaptic enzyme AChE terminates transmission at cholinergic synapses by rapidly hydrolyzing ACh. It exhibits a more selective specificity for the substrate ACh compared to BChE, and since the discovery of cholinesterases superfamily was the main focus of study due to its fundamental impact on ACh. It has been localized in many types of conducting tissues with muscle and brain as the main source of the enzyme (Jbilo et al., 1994). Identified at the neuromuscular junction (NMJ) in 1930s as the first protein of NMJ, its local expression has shown to be regulated by the motoneuron in 1937 (Rotundo, 2003). During the same years also its characterization at a central level has been elucidated (Andra et al., 1988), connected to cholinergic transmission even though there are also evidence suggesting that its functions could not only be linked to cholinergic transmission (Appleyard, 1994).

2.2 The Butyrylcholinesterase

The first evidence of the existence of BChE dates back to 1932, when it was isolated an enzymatic activity catalyzing the hydrolysis of esters of choline (Stedman et al., 1932). The isolated product which has been identified as the responsible for the hydrolytic action, was also demonstrated to be the responsible for the catalysis of the hydrolysis of non-choline esters. Subsequently, it was also established that there were two types of enzymes responsible for the hydrolytic activity, both of them contained into the isolated product. One enzyme displayed specificity for the neurotransmitter ACh (Alles and Hawes, 1944), while the other catalyzed the hydrolysis of a variety of choline and non-choline esters (Mendel and Rudney, 1943). The non-specificity of the enzyme BChE immediately came out, suggesting one of its first name, 'pseudocholinesterase', while the other enzyme which showed specificity towards the substrate ACh was called 'specific' cholinesterase or Acetylcholinesterase, AChE. Despite the correlation with ACh as one of the most important substrates for BChE for its physiological relevance, one of BChE specific substrates is butyrylcholine, but although exogenous butyrylcholine has been shown to modulate intrinsic cardiac neuron activity in canines (Darvesh et al., 2004), no synapses in higher vertebrates use butyrylcholine as a neurotransmitter. The name Butyrylcholinesterase was assigned in 1989 by the Human Gene Nomenclature committee directed by Phyllis J. McAlpine. The abbreviation for the gene is BCHE and for the protein is BChE. French laboratories introduced the abbreviation BuChE; *bûche* which means *"log"* in French, while the older literature employs the names serum cholinesterase, plasma cholinesterase, nonspecific cholinesterase, pseudocholinesterase, butyrylcholinesterase, and acylcholine acylhydrolase. In contrast to the long-established and well-defined role of AChE in regulating cholinergic signaling, a true physiological function for BChE remained elusive over many decades. After its discovery, BChE was considered an interesting molecule in the field of anesthesia (Davis et al., 1997; Pedersen and Jensen, 1994) since after the introduction of the muscle relaxant succinylcholine (Evans et al., 1952), it was found out to end the effects of that drug. In particular, it was observed that some patients who were not able to clear succinylcholine from their system and experienced prolonged, life-threatening apnea had unusual variants of BChE with reduced catalytic activity (Kalow and Staron, 1957). The interest for BChE grew noticeably in '90s also due to its roles as a detoxicant molecule towards toxic nerve agents such as soman (Broomfield et al., 1991), organophosphorus compounds such as sarin and tabun, which in those period were unlawfully developed as a chemical weapon by various nations (Broomfield et al., 1991). The amounts of studies performed corroborated the idea that this pseudocholinesterase

had a larger spectrum of substrates than esters of choline (Ahmed et al., 2007; Raveh et al., 1993) . The broader active site of BChE, in fact, enables it to act on more diverse substrates, allowing the enzyme to be involved in many physiological processes and diseases. Among the other compounds, important hydrolytic activity of BChE on esters includes cocaine (Xie et al., 1999), acetylsalicylic acid (Zhou et al., 2013) and heroine (Qiao et al., 2014). More recently, numerous studies have been conducted regarding BChE versatility as a pharmacological tool. It was included in pharmacological studies on Alzheimer disease, since it was found in β-amyloid plaques, which originate in brain during the development of the disease (Gomez-Ramos and Moran, 1997). Moreover, some other implications of the enzyme were related to the Parkinson disease, since high levels of BChE have been found in patients suffering from dementia, while not in healthy subjects (Ruberg et al., 1986). Animal models have been used as diffused tools to study this enzyme and its related functions. A knock-out mouse model was proposed and realized by Li et al. in 2006 (Li et al., 2006) and it was observed that BChE *knockout* mice with no BChE expression appeared perfectly healthy (Duysen et al., 2009); in particular, they showed no apparent change in motor, autonomic or cognitive function. Under casual observation they were indistinguishable from wild-type mice. Moreover, there are isolated human populations who have been identified as completely lacking a functional BCHE gene, but again, they exhibit a normal phenotype. Their only physiological difference from subjects with a normal phenotype is an elevated risk when exposed to bioactive esters or ester-type muscle relaxants in the clinic (Lockridge et al., 2016; Manoharan et al., 2007). Initially, BChE was considered to lack an important function apart from serving as a "backup" for AChE in regard to neurotransmission and as a modestly protective bioscavenger towards bioactive esters. Despite this, more recently it was discovered and proposed a new challenging and interesting role for that enzyme linked to obesity and appetite regulation: the hydrolysis of the neuropeptide gut hormone, ghrelin (Chen et al., 2016; De Vriese et al., 2004).

2.2.1 BChE molecular forms

As well as for AChE, also BChE exists in different molecular forms, including monomers and oligomers with identical catalytic subunits (Massoulie and Bon, 1982; Massoulie et al., 1993) (Chatonnet and Lockridge, 1989) (figure 2).

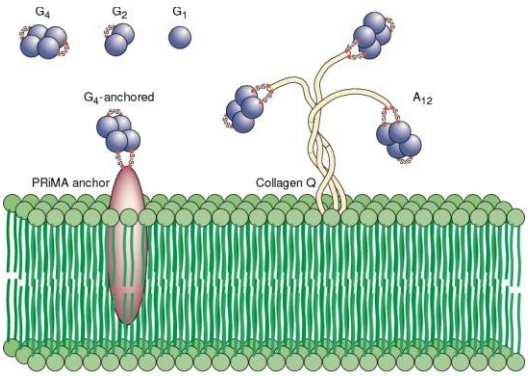

Figure 2 | *Molecular forms of BChE*. G1: monomeric soluble globular form. G 2: dimeric soluble globular form. G4: tetrameric soluble globular form. The G 4-anchored form is a tetrameric globular form membrane-bound owing to its interaction with the proline-rich membrane anchor (PRiMA). A12: asymmetric form with three tetrameric forms anchored to the membrane by a collagen tail (collagen Q). (Darvesh et al., 2003)

The symmetric monomeric form is called the G1 (globular) form. The dimeric form, named the G2 form, consists of two monomers linked by a disulfide bridge formed between the cysteine 571 residues of each monomer (Lockridge et al., 1987). Two G2 forms are kept together by hydrophobic interactions to form a tetramer, the G4 globular form (Lockridge et al., 1987). The G1, G2 and G4 molecular forms are all symmetrical, hydrophilic, globular forms, and are found primarily in soluble form. Globular BChE also occurs in asymmetric, membrane-bound forms that are amphiphilic and consist of tetramers (G4) anchored to membranes by a protein anchor: the proline-rich membrane anchor (PRiMA)(Massoulie, 2002; Perrier et al., 2002). In addition, there are other forms of asymmetric, collagen-linked BChE that consist of tetramers of catalytic subunits that are attached to membranes by a triple helical, non-catalytic, collagen anchor. The tetramer in the collagen-tailed form is called A4, two tetramers make up the A8 form, and three tetramers make up the A12 form (Massoulie et al., 1993). The collagen which composes these heteromeric multimers of BChE — collagen Q — has an amino-terminal proline-rich attachment domain that promotes assembly of these structures in the extracellular matrix (Bon et al., 1997).Not so much information have been reported regarding mouse BChE molecular forms. The identified and recognized molecular forms in mouse are G1, G2 and G4 and the prevalence of one or another is dependent on the anatomical district in which BChE is localized (Gomez et al., 1999; Massoulie, 2002; Shan, 2004). The principal

molecular forms identified in mouse and rat intestine are the G2 and G4 forms. The G4 form is considered to be generally a membrane-bound molecular BChE form although it is released as a soluble enzyme from nerves and is found in plasma of many mammals, while G1 form is considered to be a cytoplasmic soluble form (Sine et al., 1988). Moreover, it was showed that the epithelial cells of the rat intestine contain only globular forms of BChE (Leparoux et al., 1992). To date, there are no information available regarding gastric mouse molecular forms of the enzyme.

2.2.2 Expression of BChE in the mouse and human organs

Since it has been identified, BChE has been widely viewed as the backup enzyme for AChE in cholinergic neurotransmission due to its hydrolytic potential towards molecules containing esters, in particular, ACh. Despite their similar catalytic function, AChE and BChE have in most of cases a different tissue distribution, different substrates, inhibitor sensitivity and physiological functions. BChE was discovered as a detoxifying agent in '90s (Broomfield et al., 1991) and the interest for this molecule grew significantly so that it was started to be studied as an independent enzyme. Furthermore, its localization has begun to be clarified. Identified as an hepatic enzyme (Kean et al., 1986), liver was the organ in which was possible to find the highest amount of the molecule in mouse and humans (Gomez et al., 2000; Jbilo et al., 1994).

First works showed the presence of human BChE RNA in plasma, red blood cells, skeletal muscle and liver, followed by lung, pancreas and brain (Jbilo et al., 1994), while in rats it was early detected a pseudocholinesterase activity in plasma, liver, pancreas and adipose tissue (Oreskovic and Kunec-Vajic, 1992). Since its production is mostly related to liver, plasmatic levels of BChE are also used to detect liver damages or disease and it is known that when liver functions are altered, the levels of plasmatic BChE are higher (Pohanka, 2013). It is also known from literature that BChE levels in liver and plasma are quantitative higher in HFD conditions (Siskova et al., 2016). This evidence is also confirmed through electron microscopy technique in *ob/ob* mouse models in which have been described a more intensive staining for BChE in liver in the rough endoplasmic reticulum (Kean et al., 1986). Several studies regarding the activity of the enzyme have been carried out on plasma samples (Rudakova et al., 2011), since its hydrolytic action towards its substrates (among which ghrelin) has been supposed to happen in plasma (Chen et al., 2015; Hosoda et al., 2004). After the discovery of BChE as the responsible for the hydrolysis of the hunger hormone ghrelin, the interest for BChE as a molecule involved in regulating appetite increased. Lot of studies were carried out with the *knockout* (−/−) mouse model, which is more prone to obesity than the *wildtype* (wt)

littermate while fed with high fat diets, despite phenotypically indistinguishable from the *wt* genotype (Li et al., 2008b). In addition, BChE was also largely localized at the neuromuscular junction where it exerts an important "helping function" towards AChE in the hydrolytic process of ACh degradation (Blondet et al., 2010). Its detoxifying role was detected through the localization of the enzyme in human lung, kidney, spleen. Pseudocholinesterase was also found in rat heart, where it exerts potential cardioprotective role linked to the degradation of non-neuronal ACh in the cardiac non-neuronal cholinergic system (Kilianova et al., 2020). BChE presence in the central nervous system has also been widely studied. An important tool to study BChE distribution and functions into brain regions was the *AChE -/-* model (Mesulam et al., 2002). The study of that model revealed that both the wild-type and nullizygous mice showed BChE enzyme activity extended to all parts of the brain receiving cholinergic innervation, and, in addition, that BChE could hydrolyze the ACh surrogate acetylthiocholine. Moreover, in contrast to AChE, which is mainly of neuronal origin, BChE appeared to be primarily of glial origin (Mesulam et al., 2002). Additional studies have been carried out in mouse models and many nuclei have been described as containing BChE. In particular, a BChE activity visualized through immunohistochemical technique was shown in rat forebrain and upper brainstem (Geula and Nagykery, 2007). A complete immunohistochemical map of the diverse mouse brain region with BChE activity can be found in Darvesh, 2013 (Darvesh, 2013). Moreover, a quantitative analysis of brain BChE was carried out through imaging system in mouse (Johnson et al., 2009).

2.2.2.1 Expression of BChE in the rodents and human gastrointestinal tract

According to those new and challenging findings, studies regarding the presence and distribution of BChE in the mouse gastrointestinal tract grew. There are only few studies describing the localization of the enzyme along the gastrointestinal tract through histochemical techniques. BChE activity in mucosal cells of the intestine has been described in rodents and other animal species in 1988 by Sine and colleagues (Sine et al., 1988), who also identified in human through immunohistochemistry the presence of BChE at the apex of the villi. They also measured the enzymatic activity of BChE and characterized its molecular forms along the intestinal tract demonstrating that the prevalent molecular form was the globular G4 form (Sine et al., 1991), while BChE activity in its G2 molecular form was detected in mucosal cells of rat intestine (Sine and Colas, 1996). Electron microscopy studies revealed the localization of the enzyme in intestinal epithelial cells of rats (L'Hermite et al., 1996). The analysis of the activity of BChE in starved and refed rats in the intestinal epithelial cells

also revealed an increased activity of the enzyme after starvation in the jejunum and the same level of activity was maintained after refeeding; much higher levels of activity were described in the small intestine (duodenum, jejunum and ileum) than in the colon and the caecum (Leparoux et al., 1992). Moreover, BChE miRNA were also found in mouse gut, involved in the intestinal inflammation process (Nadorp and Soreq, 2015). As regard the more distal portion of intestine, the colon, no information about BChE distribution have been reported in mouse, while BChE has been localized for the first time in 2017 through immunohistochemical technique in the cytoplasm of human colonic epithelial crypt cells (Damm et al., 2017). In addition, BChE activity was detected also in human and mice stomach (Roivainen et al., 2004; Wang et al., 2004), but, to date, there are no evidence regarding precise localization and no morphological studies have been performed. Only one study described the presence of BChE through light and electron microscopy in the rat stomach fundic mucosa and pseudocholinesterase positive cells have been identified as enterochromaffin-like cells (ECLs) (Monga et al., 1974). Quantitative BChE expression was lastly reported in mouse stomach muscle (Worth et al., 2015).

2.3 BChE physiological functions

2.3.1 Ghrelin, BChE and Obesity

Ghrelin is a 28 amino acids peptide hormone isolated in 1999 from rat stomach and identified as a ligand for the growth hormone secretagogue receptor (GHSR), a seven transmembrane G protein coupled receptor (Kojima et al., 1999). The protein product of ghrelin gene requires post-translational modifications to generate the active form of ghrelin peptide (acyl-ghrelin). In particular, to activate its only known receptor, des-acyl ghrelin needs the attachment of a fatty acid side chain (preferably C8 or C10) to its Serine 3 residue. This kind of modification a post-translational acylation achieved by the GOAT enzyme (Ghrelin O-acyltransferase), a member of the MBOAT family (membrane bound O-acyltransferase) (Gutierrez et al., 2008; Yang et al., 2008). The octanoyl and decanoyl acylated forms of ghrelin are the optimal ligand for Ghrelin's receptor, the GHSR1a (Kirchner et al., 2009; Lim et al., 2011; Yang et al., 2008). The length of the fatty acid used for ghrelin acylation seems to be of importance for ghrelin's metabolic effects since alterations in the fatty acid length results in differential activation of GHSR1a *in vitro* and alter ghrelin's effects on food intake *in vivo* (Heppner et al., 2012). The lipids required for ghrelin activation are mostly directly derived from the ingested dietary lipids (Nishi et al., 2005), an advantage for X/A like cells which produce and secrete ghrelin (Ariyasu et al., 2001) providing approximately 80% of total circulating hormone,

located within the gastric oxyntic mucosa and which could have direct access to the diet-introduced lipids (Sakata et al., 2002). The X/A like cells are present from the base to the neck of oxyntic glands in gastric fundus (Date et al., 2000) and account for about 20% of the endocrine cell population in the oxyntic glands. They are filled with round, compact electron dense granules containing ghrelin. The stomach of rodents has two ghrelin producing cell populations: the so-called closed type, round cells containing both ghrelin and des-acyl ghrelin, and the open-type cells containing only des-acyl ghrelin (Zhao and Sakai, 2008). Apart from the stomach, the small intestine, colon (Date et al., 2000) and pancreas produce a small amount of ghrelin as well (Date et al., 2002). Until the discovery of Asprosin (Duerrschmid et al., 2017), ghrelin was the only orexigenic peptide hormone known to be produced in peripheral organs (Nakazato et al., 2001; Tschop et al., 2000). Because of its peripheral production, ghrelin is considered to be a gut-brain peptide since its action is mostly exerted at a central level.

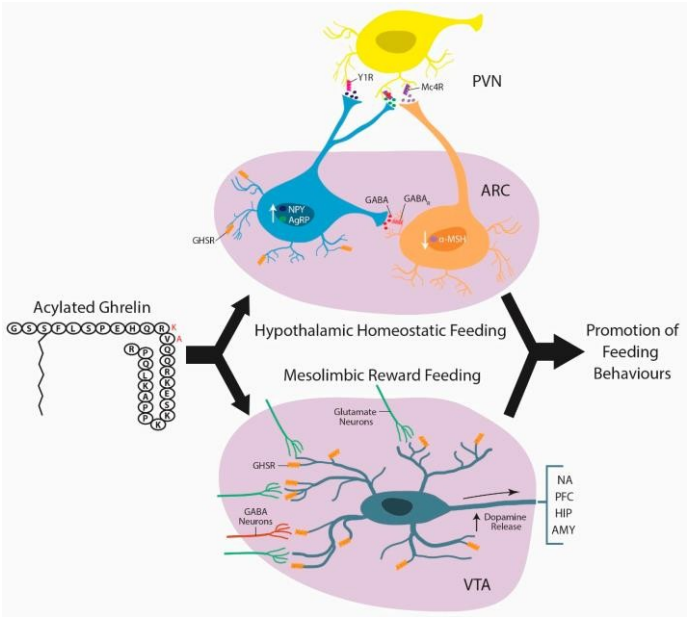

Figure 3| *The two main brain regions where acylated ghrelin is supposed to target to trigger neurocircuits which promote feeding behaviors: the arcuate nucleus (ARC) of the hypothalamus (Arkhypova et al.) and the ventral tegmental area (VTA).* It also shows ghrelin peptide sequence: human ghrelin is black amino acid sequence (red letters stand for amino acid substitutions in rodents) (Edwards and Abizaid, 2017)

Known as *hunger hormone* or *feeding peptide*, ghrelin exerts its action of regulator of the homeostatic feeding and energy balance especially in hypothalamic nuclei expressing GHSRs (arcuate, paraventricular, dorsomedial and ventromedial among the other)(figure 3)(Cowley et al., 2003; Guan et al., 1997; Shuto et al., 2001; Willesen et al., 1999). The hypothalamus, especially the arcuate nucleus (ARC), which is adjacent to the third ventricle and the median eminence (a

circumventricular organ), is an important brain region as it has receptors for and is sensitive to most circulating hormones that influence energy balance, including ghrelin (Olszewski et al., 2003). Acylated ghrelin is proposed to activate neurocircuits that promote feeding behaviors mainly in two brain regions: the ARC, and the ventral tegmental area (VTA), an extra-hypothalamic region important for the mesolimbic dopaminergic system, which controls motivational aspects of multiple behaviors, including hedonic feeding (Alvarez-Crespo et al., 2012; Kanoski et al., 2013). The circulating levels of ghrelin vary with feeding status: higher levels in the fasted state and lower post-prandially, falling to minimum levels within 1 hour after eating (Cummings et al., 2001; Tschop et al., 2001). Like ghrelin, also GOAT expression levels vary in response to feeding status and diet, following ghrelin expression pattern (Gahete et al., 2010). This discovery led to the idea that the GOAT-ghrelin system acts as a nutrient sensor informing the body of the presence of nutrients, rather than the absence, as commonly proposed (Kirchner et al., 2009) and could stress the importance of ghrelin for meal initiation and appetite regulation. The principal form of circulating ghrelin in blood is des-acyl ghrelin (McGovern-Gooch et al., 2016). Although there are speculations about a potential receptor for it, located in the cardiovascular system (Baldanzi et al., 2002), to date no receptor for des-acyl ghrelin has been identified. At the same time, it is not clear if des-acyl ghrelin could cross the blood brain barrier or if it is the acylated form that reaches the brain. There is also the hypothesis that all ghrelin amount in circulation is acylated and that des-acyl ghrelin may be an artifact of sample handling (Blatnik et al., 2012). What is clear and quite recently discovered instead, is the fact that the action of ghrelin is ended by carboxylesterase, in particular BChE, at a plasmatic level (De Vriese et al., 2004) . Body weight is controlled by the central nervous system involving expecially the hypothalamic area, where peripheral hormonal signals are integrated to regulate food intake and energy expenditure. Acyl-ghrelin is a key hormone in the field of energy balance playing an important role in energy homeostasis and metabolism. In 2004 it was first reported to be associated with BChE by a study of De Vriese and coworkers (De Vriese et al., 2004) . They pointed out that BChE is responsible for ghrelin hydrolysis removing the octanoyl group essential for ghrelin interaction with its receptor GHSR1a, thus for its activity. In the following years, an increasing number of studies proved it. Among the others, Dorling et al., demonstrated that lower plasma BChE activity is associated with higher rate of ghrelin/des-acyl ghrelin (Dorling et al., 2019). Taking into consideration the hydrolysis impact of BChE on ghrelin, and the influences of this on increasing appetite and regulating energy expenditure, it is possible to affirm that BChE might be involved in the regulation of energy balance. The relevance of that enzyme in the field of metabolic

disorders and regulation of appetite grew significantly. Mostly related to obesogenic environments with abundant calorie-dense food, passive entertainment, and little physical activity, obesity affects over one third of the world's adult population which has been shown to be overweight or obese and the prevalence is rising fastly (Ng et al., 2014). Numerous studies carried out to date, aimed to control that condition, employ genetically modified animal models as tools to analyze the role of BChE in the complicate physiological system (Chen et al., 2016; Duysen and Lockridge, 2011; Hrabovska et al., 2010; Li et al., 2008a; Li et al., 2006; Wang et al., 2004). Chen et al. reported that *BChE-KO* mice show the trend to reach obesity states easier than *wild type* mice, especially when being treated with high-fat diets (Chen et al., 2016). They also pointed out that peripheral administration of BChE in *BChE-KO* mice restored nearly all aspects of the *wild type* mice, confirming the effect of BChE on fat metabolism and accumulation (Chen et al., 2016). However, although peripheral expression of BChE inhibited overeating and overweight of these KO mice and maintained them lean and healthy, they still developed hyperinsulinemia and insulin resistance (Chen et al., 2016). Additional experiments found that these diet-induced metabolic lesions could be rescued through the expression of BChE both in central and circulating systems (Chen et al., 2017b), suggesting a central role of BChE in regulating insulin and glucose homeostasis. Mood-related behavior of mice, such as anxiety, has also been proved to be influenced by ghrelin (Spencer et al., 2012). Considering the role of BChE as a "ghrelin hydrolase," BChE could also be seen as an emotional behavior-influencing factor. For instance, Chen et al.(Chen et al., 2015) reported that *BChE-KO* mice showed much more aggressive behaviors than *WT* mice, and elevated plasma BChE could attenuate the aggression, corroborating the idea that ghrelin hydrolysis came from BChE. Another interesting finding is also the link between ghrelin, BChE, Alzheimer disease (AD) and metabolic disfunction (Procaccini et al., 2016). It was demonstrated that some disease developing frequently in middle age, including hypertension, diabetes, metabolic syndrome and overweight, are also risk factors of AD in the older (Vanhanen et al., 2006). Weight loss is a common symptom in AD patients, observed in more than 80% of patients and in most of cases manifests even earlier than impairments of cognitive functions (Soto et al., 2012). In 2019, Ishii and coworkers (Ishii et al., 2019) showed the relationship between early weight loss of AD patients and the arcuate neuropeptide Y neurons, related to food-intake. It was also shown that undernutrition and weight loss states could contribute to worse cognitive impairment (Vellas et al., 2005). Recent studies have shown that the effects of a nonselective ChE inhibitor rivastigmine or donepezil could improve appetite of AD patients relating to the different levels of ghrelin. According to these studies,

selective AChE inhibitor donepezil did not show such effects, which might explain the possible role of BChE in elevating appetite through its action on ghrelin hormone. AD is closely related to obesity and raising appetite of AD patients could be helpful to improve their nutritional status. In addition, since it might be difficult for moderate and severe AD patients to balance and pay attention to their diet, improvement of appetite could also reduce the pressures on caregivers. Taking into consideration the effect of nonselective ChE inhibitor rivastigmine on elevating appetite and the ability of ghrelin to stimulate food-intake, it can be hypothesized a role for BChE in improving diet of AD patients, even if this aspect needs further in-dept studying.

2.3.2 BChE and gastrointestinal stem cells

2.3.2.1 Gastric cytotypes and stem cells

The mouse stomach can be divided into three regions: the fundus, the corpus and the antrum, which opens into the duodenum. Each of these parts are lined by a glandular epithelium which is organized into multiple gastric units composed of three main structural elements: a planar surface epithelium, tubular invaginations of the surface epithelium called pits, and tubular extensions of the pits called glands. The precise structure and composition of these gastric units varies in different anatomical regions of the stomach (Lee et al., 1982). Many mammals, especially rodents, instead of a glandular fundus, possess a large forestomach lined with simple columnar epithelium, the same kind of epithelium which composes the esophagus. The stomach shares lots of features with the intestine, including a common endodermal origin and a constantly renewing epithelium. In both organs, cell renewal is fueled from stem cell populations located in pockets within the epithelium. The gastric unit stem cell zone has been assigned to a region just above the neck of the gland, named isthmus, which shows a high cellular turnover and which is the location of a population of mostly quiescent stem cells (Karam and Leblond, 1993). In adults, each gastric unit in the pyloric and corpus region is considered to be functionally monoclonal, with the cellular progeny being derived from a single stem cell which is thought to maintain a small population of clonal, multipotent stem cells through symmetric division (McDonald et al., 2008). Daughter cells generated by divisions of these multipotent stem cells subsequently exit the stem cell niche and differentiate to generate the various epithelial cell lineages as they migrate bidirectionally towards the pit or the gland (Bjerknes and Cheng, 2002). Their activity is under the control of Notch signaling (Kim and Shivdasani, 2011), as inhibition of Notch leads to differentiation of proliferating cells in the isthmus (Faure et al., 2015). Different cell types follow different migratory ways from the isthmus. In the corpus of the stomach,

the mucus secreting pit cells migrate up to the pit and surface, whereas cells in the zymogenic lineage migrate down into the glands. Parietal, enteroendocrine, and caveolated cells migrate in both directions (Karam, 1993; Lee and Leblond, 1985). Besides the isthmus region, a second region of stem cell activity has been identified in the stomach. The intestinal stem cell marker Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) can be found at the bottom of the stomach glands. Lineage tracing experiments proved that these cells could generate entire glands in the antrum/pyloric region (Barker et al., 2010), but while in the antrum this cell population is frequently dividing, the corpus does not show prominent proliferative activity at gland bottoms. It is yet to clarify through which mechanisms the isthmus and gland bottom stem cells compete in the homeostasis and regeneration of the glands but, as well as in the intestinal epithelium, mesenchymal signaling is involved in the maintenance of gastric homeostasis.

2.3.2.2 Intestinal cytotypes and stem cells

In the gastrointestinal tract, multipotent stem cells, housed in specific 'niches' within intestinal crypts, give rise to all cell types through precursor cells (Umar, 2010) The intestinal epithelium consists of two different compartments: the proliferative crypts of Lieberkühn and long finger-shaped projections called villi, continuous structures that contain a single columnar layer of fully differentiated epithelial cells no longer capable of dividing. The stem cell niche in the small intestine is composed of stem cells and Paneth cells, and it is surrounded by mesenchymal cells at the crypt bottom (Sato et al., 2011; Tian et al., 2011). This provides a peculiar microenvironment that constitutes a constantly renewing dynamic system along the crypt–villus axis. The intestine, in fact, requires a constant supply of differentiated cells to perform its numerous functions, including digestion and absorption of food and nutrients, involving multiple key signals from the surrounding niche (Takahashi and Shiraishi, 2020). Intestinal stem cells (ISCs) in the crypts divide continuously to fuel this high turnover, originating another stem cell for self-renewal, or a progenitor cell that enters the trans-amplifying (TA) compartment to rapidly divide before terminal differentiation (Takahashi and Shiraishi, 2020). TA cells rapidly undergo 4 −5 cycles of division, move out of the crypt toward the villi, and differentiate into enterocytes, the most significant cell type of the intestinal epithelium, goblet cells, or enteroendocrine cells (Clevers, 2013). These differentiated cells continue to move toward the apex of the villi and die after 3 −5 days. An exception is represented by Paneth cells, which also derive from TA cells but migrate down toward the crypt and are renewed every 3 −6 weeks (Clevers, 2013; Sato et al., 2011). In the large intestine, there are numerous crypts, with

regional variations in size and cell types. Two major types of progenitors exist within the intestinal epithelium: the absorptive and secretory progenitors. Absorptive progenitors differentiate into enterocytes, whereas secretory progenitors give rise to Paneth cells, enteroendocrine cells, tuft cells and goblet cells (Clevers, 2013). Four main cell-lineages characterize the intestinal epithelium: the columnar cells, the mucin-secreting cells, endocrine cells, and, in the small intestine, the Paneth cells. Columnar cells are the most abundant epithelial cells, with apical microvilli, called enterocytes in the small intestine and colonocytes in the large intestine, while mucin-secreting cells, or 'goblet' cells, are so called because of their abundant mucus granules producing a swollen theca, so, a 'goblet-shaped' cell. They are interspersed throughout the colonic epithelium and secrete mucus into the intestinal lumen. Endocrine or 'neuroendocrine' or 'enteroendocrine' cells are an abundant cell population distributed throughout the intestinal epithelium. In the small intestine, these cells are more common in the crypts than the villi. They secrete peptide hormones in an endocrine or paracrine manner from the dense core or neurosecretory granules. Paneth cells are located at the crypt base of the small intestine and contain large apical secretory granules.

2.3.2.3 Cholinergic activation of gastro-intestinal stem cells

The presence of components of the cholinergic system outside of the nervous system was first reported in 1978 by Sastry and Sadavongvivad (Sastry and Sadavongvivad, 1978); interestingly, the non-neuronal ACh has been also localized in epithelial cells of the small and large intestines of rats and humans (Klapproth et al., 1997) where it is predicted to function as a trophic molecule controlling the growth and differentiation of crypt–villus organoids. Moreover, ACh is involved in both the proliferation and differentiation of Lgr5+ ISCs in the small intestine by binding to muscarinic ACh receptors (mAChRs) in crypt-villus organoids (Takahashi et al., 2014). It has also been shown that muscarinic and nicotinic ACh receptors are involved in the proliferation of mouse embryonic and induced pluripotent stem cells (Landgraf et al., 2010; Paraoanu et al., 2007), suggesting the presence of a *cholinergic niche* that affects stem cell behavior (Takahashi, 2021). Schofield (Schofield, 1978) was the first to hypothesize the existence of a microenvironment that is required for the maintenance of stem cells using hematopoietic stem cells calling such a region *"niche"* and defining it as a subset of tissue cells and extracellular substrates that can indefinitely maintain stem cells and control their self-renewal and progenitor cell production *in vivo*. Homeostasis at the stem cell level must be regulated to maintain a close balance between self-renewal and differentiation. Enhanced self-renewal, on one hand, expands the stem cell pool, increasing the total number of

target cells that can undergo oncogenic transformation and increasing the risk of tumorigenesis. Decreased self-renewal, on the other hand, decreases the stem cell pool and compromises regeneration. The accumulation of mutations in adult stem cells that occurs during aging carries the dual risk: compromised regeneration and enhanced oncogenic transformation. According to cancer statistics, stomach cancer and colorectal cancer are the 3rd and 4th most frequent reason for cancer associated death worldwide, accounting each one for around 700.000 victims per year (Torre et al., 2015). The gastrointestinal tract, a continuous structure composed by the stomach, the small-intestine and the large-intestine is characterized by a self-renewing epithelium replaced through adult stem cells residing at the bottom of the intestinal crypt and gastric glands (figure 4). These organs in fact are constantly in contact with nutrients, metabolites and resident bacteria, which on the one hand are indispensable for health, but on the other cause constant damage to the epithelium, including genetic alterations (Alonso and Yilmaz, 2018). The activity and proliferation of stem cells are strongly dependent on complex signaling pathways involving other crypt/gland cells as well as surrounding stromal cells.

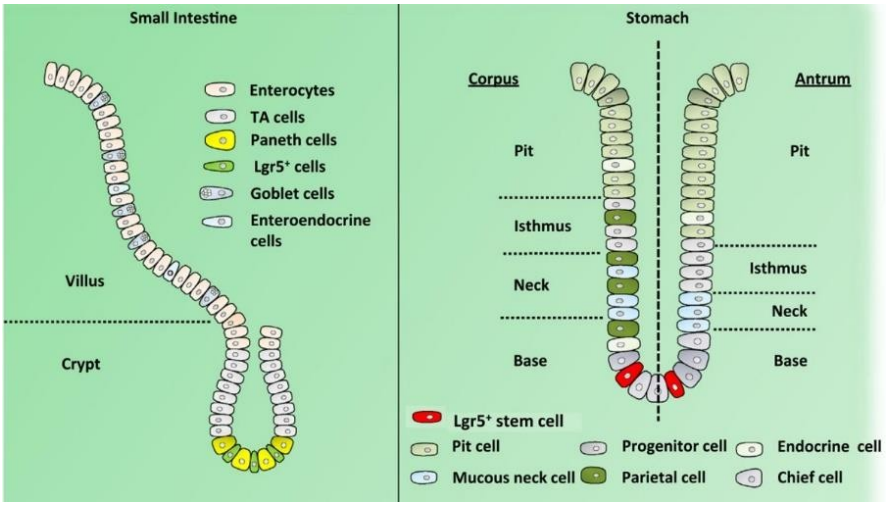

Figure 4| *Structure of intestinal and gastric unit.* (Merker et al., 2016)

The simultaneous stimulation of both ACh receptors and the crosstalk between these receptors may control the signals emanating from epithelial cells and contribute to repair following tissue injury. Signaling through the M3 muscarinic receptor and α2β4 nicotinic receptor work together to maintain the homeostasis of intestinal epithelial cell growth and differentiation, following modifications of the cholinergic intestinal niche. Muscarinic receptors (M1–M5) are G-protein-coupled receptors responsible of mucosal ion transport (Cameron and Perdue, 2007), epithelial proliferation (Gross et al., 2012), immune-defensive mechanisms (Labed et al., 2018), as well as

cholinergic neurotransmission at effector cells. M3, a subtype widely expressed throughout the gastrointestinal tract, couples to G αq/11 to increase intracellular calcium via the activation of phospholipase C signaling and inositol phosphate formation. As its pathway outcomes are fundamental component of cell functions, M3 signaling is considered to be responsible to alter cell function, including proliferation and differentiation (Slack, 2000; Takahashi et al., 2014). How ISCs proliferation, differentiation, and maintenance are regulated, and which signals are required for tissue maintenance, are well established (Takahashi and Shiraishi, 2020), but little is known about the regulation of these pathways in vivo. Moreover, Middelhoff and coworkers, showed a novel interaction between M3 signaling and Lgr5+ ISC maintenance (Middelhoff et al., 2020) demonstrating how the deletion of M3 reduced the number of Lgr5+ ISCs. ACh through its binding to M1, M2, and M3 receptors has been suggested to activate epidermal growth factor regulatory pathways (Stirnweiss et al., 2006; Tsai et al., 1997) supporting the signaling mechanisms underlying the proliferation and differentiation of Lgr5+ ISCs. In addition, physiological homeostasis is also regulated by nAChR which play a pivotal role in other regulatory processes such as regulation of epithelial cell growth, migration, differentiation, and inflammation processes in various mammalian non-neuronal cells (Takahashi, 2020; Wessler and Kirkpatrick, 2008). Nicotinic AChRs use ACh as an endogenous ligand and nicotine as agonist for signaling activation. Toshio Takahashi and colleagues have shown that nicotine increases organoid growth and differentiation (Takahashi et al., 2018). In contrast, mecamylamine, a non-selective, non-competitive antagonist of the nAChrs, has an antagonistic effect compared with nicotine (Takahashi et al., 2018). In addition, it has been demonstrated through immunohistochemical analysis the existence of α2β4 receptor subtype in Paneth cells, in the crypt region, giving potentially novel functions such as the regulation of stem cell proliferation and differentiation to nAChRs (Takahashi et al., 2018). It has also been demonstrated that deficiency in the β4 subunit causes a decrease in crypt size and ISC proliferation and differentiation (Takahashi et al., 2020). Furthermore, also the proliferation of the gastric stem cell niches is under the control of ACh. It has been demonstrated in fact that gastrointestinal hormones, such as gastrin and ACh, play unique roles in the antral stem cell niche. ACh generated from tuft cells in gastric epithelium regulates gastric epithelial proliferation and regeneration, as well as the clonal expansion of $Lgr5^{+}$stem cells via the muscarinic receptor subtype 3 (M3R) (Hayakawa et al., 2017; Zhao et al., 2014). To corroborate the role of ACh in proliferation, it has also been studied three separate mouse models of gastric cancer. Each of them has shown a markedly reduced tumor incidence and progression after surgical or pharmacological denervation of the

stomach which, interestingly, regarded only the denervated portion. This evidence suggests that vagal innervation contributes to gastric tumorigenesis via M3R-mediated Wnt signaling in the stem cells, and also that denervation might represent a feasible strategy for the control of gastric cancer (Zhao et al., 2014).

From all those evidences emerge that ACh is one of the major signaling molecules which is able to regulate and/or control cell functions in the mouse gastrointestinal tract.

3 AIMS OF THE PROJECT

BChE is an hydrolytic enzyme studied since many years related to its action towards the neurotransmitter ACh, but its precise physiological function has not been uniquely defined yet.

The recent challenging discovery of ghrelin as one of its suitable substrates, grew considerably the interest for this enzyme in the field of feeding and appetite regulation, reflecting the will of elucidating its precise physiological function. Despite some aspects regarding BChE enzymatic activity have already been elucidated, the distribution of BChE was not deeply investigated yet. Starting from this gap in literature, we performed a morphological study on the gastrointestinal distribution of this enzyme identifying the specific BChE-expressing cytotypes. Hence, the data we propose in this thesis work could be helpful in order to go better in depth in possible BChE physiological roles.

4 MATERIAL & METHODS

4.1 Animals and experimental conditions

Male C57BL/6 mice were purchased from Charles River Laboratories (Calco, Italy), housed individually and kept under constant environmental conditions in a 12 h light/dark cycle at 22 °C. They had free access to standard chow diet and water and handling was limited to cage cleaning. They were killed for experimental procedures at 12-14 weeks of age. All mice initially received a standard diet; when they were 4 weeks old, n=6 mice were switched to a high-fat diet (HFD; Charles River; 50 kJ% from fat, 30 kJ% from carbohydrates and 20 kJ% from proteins) for 10 weeks, while n=6 were maintained on standard chow diet. Before being killed they were intraperitoneally euthanized with an overdose of anesthetic 2,2,2-tribromoethanol (Avertin) (Sigma-Aldrich, Saint Louis, MO, USA) and immediately transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 5 minutes. Gastro-intestinal organs (salivary glands, esophagus, stomach, liver, pancreas, spleen and intestine) were removed and processed for either morphological or molecular analyses. All efforts were made to minimize animal suffering and to reduce the number of animals used. Experiments were carried out in accordance with Council Directive 2010/63/UE and all experiments were approved by the Italian Health Ministry (authorization no. 405/2018-PR).

4.2 Tissue processing

For morphological analyses, after anaesthetizing, mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, gastro-intestinal organs were removed. All depots were dissected using a Zeiss OPI1 surgical microscope (Carl Zeiss, Oberkochen, Germany) and further fixed by immersion in 4% paraformaldehyde in PB overnight at 4°C. Stomachs were immediately divided into two parts longitudinally using a lancet, washed in physiological solution and post-fixed, as well, in 4% paraformaldehyde for 24 hours. All depots were washed briefly in PB before starting the paraffin embedding processes in which they were dehydrated in ethanol (several passages from alcohol 75°, 96° and 100°), cleared in xylene, and embedded in paraffin. Serial paraffin sections (3 µm thick) were obtained from each depot, placed on glass slides, and dried for at least 24h before being used. Brain specimens were carefully removed from the skull, postfixed in the same fixative solution for 24 h at 4°C and washed in PB. Free-floating coronal sections (40-µm-thick) were cut with a Leica VT1200S vibratome (Leica Microsystems,

Vienna, Austria) and kept in phosphate buffered saline (PBS), pH 7.4, at 4°C until use. For molecular biology analyses, mice were anaesthetized with 2,2,2-tribromoethanol (Avertin) and euthanized by cervical dislocation. Gastro-intestinal organs were rapidly removed, snap-frozen in liquid nitrogen and stored at -80°C until use.

4.3 Immunohistochemistry and light microscopy

Standard peroxidase immunohistochemical (IHC) staining methods were used. Serial paraffin sections (3 µm thick) were obtained from each depot, placed on glass slides, and dried. Alternate sections were used for hematoxylin and eosin staining to assess morphology, and for immunohistochemical procedures to evaluate tissue protein expression and localization. Briefly: paraffin sections were dewaxed through sequential passages in xylene, alcohol 100°, 96°, 75°. Treated with H_2O_2 (3% in dH2O; 5 min) to block endogenous peroxidase, tissue sections were rinsed twice in PBS and treated with normal serum blocking solution (2% in PBS; 20 min at room temperature in humid chamber) to block non-specific binding of the secondary antibody. Next, primary antibody diluted in PBS was applied to the sections (overnight at 4°C, in a humid chamber). The next day, sections were rinsed in PBS and biotinylated secondary antibody was added (1:200 in PBS; 30 min at room temperature), rinsed again in PBS and incubated in avidin-biotin peroxidase complex (in PBS; 60 min at room temperature) (VECTASTAIN Elite ABC Kit, Peroxidase (Standard), Vector, Burlingame, CA, USA). Sections were washed again in PBS several times and finally incubated in 3,3'- diaminobenzidine tetrahydrochloride (ImmPACT DAB Substrate kit, Peroxidase, Vector, 1 minute). After immunohistochemical staining, sections were counterstained with hematoxylin, dehydrated in ethanol, and mounted with Eukitt® mounting medium (Sigma-Aldrich, Saint Louis, MO, USA) and coverslips. For each staining procedure, a negative control, in which the primary antibody was omitted, was added to check non-specific staining. For brain sections, a different protocol was applied. Free-floating coronal sections (40-µm-thick) were cut with a Leica VT1200S vibratome (Leica Microsystems, Vienna, Austria) and kept in phosphate buffered saline (PBS), pH 7.4, at 4°C until use. The sections were reacted with 0.3% H2O2 (in dH2O; 30 min) to block endogenous peroxidase, rinsed with PBS and incubated with 3% normal serum blocking solution (in PBS; 60 min at room temperature). Then they were incubated with the primary antibody in PBS, overnight at 4°C in humid chamber. After a thorough rinse in PBS, sections were incubated in 1:200 v/v biotinylated secondary antibody solution (in PBS; 30 min), rinsed in PBS and incubated in avidin-biotin peroxidase complex (ABC Elite PK6100, Vector), washed several times in PBS and finally

incubated in 3,3' diaminobenzidine tetrahydrochloride (0.05% in 0.05 M Tris with 0.03% H2O2; 5 min). After immunohistochemical staining, sections were mounted on slides, air-dried, dehydrated in ethanol, cleared with xylene and covered with Eukitt® mounting medium (Sigma-Aldrich) and coverslips. Staining was not detected when the primary antibody was omitted. The IHC staining reactions were analyzed using a Nikon Eclipse E600 microscope (Nikon; Sesto Fiorentino, Florence, Italy), with a video camera fitted to the microscope to obtain images.

4.4 Immunofluorescence and confocal microscopy

For double labelling studies we used paraffin embedded sections (3 µm thick). After dewaxing in an alcohol scale (from xylene to alcohol 75°), tissues were washed once in normal PBS then in 0,1% PBS-Tween and incubated with an antigen retrieval solution (Histo-VT-One, PH 9, Nacalai Tesque Product) for 40 minutes at 70°C. After three washes in 0,1% PBS-Tween, tissues were incubated with an appropriate blocking solution (Blocking One Histo- Nacali Tesque Product) just covering them with enough drops of the product as it is, at room temperature for 40 minutes. Once tapped off the blocking solution and washed slides with 0,1% PBS-Tween, primary antibody were applied (diluted in 1% BSA-PBS) and incubated overnight at 4°C. After removal of primary antibody and three washes in 0,1% PBS-Tween, secondary antibody diluted in 1% BSA-PBS were applied (1:400, Alexa Fluor, Invitrogen, Carlsbad, CA, USA) for 30 minutes at room temperature in humid chamber with no light. All the steps after secondary antibody were done in no light conditions. Other three washes to remove the excess of antibody in 0,1%PBS -Tween were performed, then To-Pro 3 (1:3000) was applied for 10-15 minutes for nuclei staining. After rinsing once with 1% PBS-Tween, slides were mounted with coverslips and Vectashield mounting medium (Vector). Sections were viewed under a motorized Leica DM6000 microscope at different magnifications. Fluorescence was detected with a Leica TCS-SL spectral confocal microscope (Leica Microsystems, Buccinasco, MI, Italy) equipped with an Argon and He/Ne mixed gas laser. Fluorophores were excited with the 488 nm, 543 nm and 649 nm lines and imaged separately. Images (1024 x 1024 pixels) were obtained sequentially from two channels using a confocal pinhole of 1.1200 and stored as TIFF files. Brightness and contrast of the final images were adjusted using Photoshop 6 (Adobe Systems, Mountain View, CA, USA).

4.5 Western blotting

Proteins from liver lysates were isolated from the phenol-ethanol supernatant obtained using homemade RIPA buffer. Soluble proteins were quantified using a Bradford protein assay (Bio-Rad, Richmond, CA, USA) and equal amounts of proteins were loaded onto homemade polyacrylamide

gels, proteins were seperated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes using a trans-blot turbo transfer system (Bio-Rad). Membranes were incubated in Every Blot Blocking Buffer (Bio-Rad) (5 minutes, room temperature) to block non specific binding of the antibodies, and incubated with the primary antibody (see table 1) (in TBS-T; overnight, 4°C). Membranes were washed thoroughly with TBS-T and incubated with the secondary antibody (in TBS-T, 2 h) Membranes were developed using clarity ECL substrate (Bio-Rad). Protein levels were assessed by densitometric analysis using a Chemidoc imaging system and ImageLab software (Bio-Rad). Results were expressed as fold changes in relative protein expression compared with the control group.

4.6 Statistical Analysis

Data were analysed using GraphPad PRISM (V8). Data are presented as mean ± SEM , and the threshold for significance was $p < 0.05$.

4.7 Antibody table

The different primary and secondary antibodies used in this study are shown in table 1 and 2 respectively.

Table 1| *Primary antibodies used for immunohistochemistry (IHC), immunofluorescence (IF) and western blotting (WB) experiments.*

Marker	Host/isotype	IHC	IF	WB	Manufacturer
Anti- Mouse/Rat Butyrylcholinesterase (BChE)	Goat/IgG	1:1000	1:400	1:500	AF-9024, R&D systems, Biotechne Brand, Minneapolis, MN, USA
anti- Human/mouse Ghrelin	Rat/IgG	1:800	1:400		MAB-8200, R&D systems, Biotechne
Anti- H+/K+- ATPase	Mouse/IgG	1:1600	1:1000		sc-374094, Santa Cruz Biotech (Santa Cruz,CA, USA)
Anti- Gastric Intrinsic Factor (GIF)	Rabbit/IgG	1:800	1:1500		PA5-87282, Invitrogen, Thermo Fisher Scientific
Anti- Chromogranin-A (Chr-A)	Mouse/IgG	1:800	1:400		sc-393941, Santa Cruz Biotech

Anti-Lysozime	Rabbit/ IgG	1:100	1:80		MA5-32154, Invitrogen, Thermo Fisher Scientific
Anti Olfm4 (D6Y5A) XP	Rabbit/ IgG	1:400	1:150		#39141; Cell Signaling Technlogy; Danvers, Massachusetts,USA
Keratin 17/19	Rabbit/ IgG		1:200		#12434; Cell Signaling Technlogy
β-actin	Mouse/IgG			1:200	Sc-477778, Santa Cruz Biotech

Table 2|*Secondary antibodies used for immunohistochemistry (IHC), immunofluorescence (IF) and western blotting (WB) experiments.*

Host	Application	Dilution	Manufacturer
anti-goat IgG, Alexa Fuor™ 488	IF	1:400	A11055, Invitrogen, Thermo Fisher Scientific
anti-mouse IgG, Alexa Fuor™ 488	IF	1:400	A21202, Invitrogen, Thermo Fisher Scientific
anti-rabbit IgG, Alexa Fuor™ 488	IF	1:400	A21206, Invitrogen, Thermo Fisher Scientific
anti-rat IgG, Alexa Fuor™ 488	IF	1:400	A21208, Invitrogen, Thermo Fisher Scientific
anti-mouse IgG, Alexa Fuor™ 555	IF	1:400	A31357, Invitrogen, Thermo Fisher Scientific
anti-goat IgG, Alexa Fuor™ 555	IF	1:400	A21432, Invitrogen, Thermo Fisher Scientific
anti-goat IgG, Peroxidase-labeled	WB	1:1000	Invitrogen, Thermo Fisher Scientific
anti-mouse IgG, Peroxidase-labeled	WB	1:5000	715-036-150, Jackson ImmunoResearch
anti-goat IgG, Biotinylated	IHC	1:200	BA-5000, Invitrogen, Thermo Fisher Scientific
anti-rat IgG, Biotinylated	IHC	1:200	BA-4001, Invitrogen, Thermo Fisher Scientific
anti-mouse IgG, Biotinylated	IHC	1:200	BA-2000, Invitrogen, Thermo Fisher Scientific
anti-rabbit IgG, Biotinylated	IHC	1:200	BA-1000, Invitrogen, Thermo Fisher Scientific

5 RESULTS

5.1 Primary antibody validation and positive controls

For morphological studies of the BChE distribution along the mouse gastro-intestinal organs we used an anti-BChE antibody, as described in material and methods section. Its specificity has been validated in control tissues which are known from literature to have high expression of the target protein: the liver and the brain.

5.1.1 Distribution of BChE in the liver under normal and high fat diet conditions

BChE is an α-glycoprotein synthesized in the liver. Parenchymal hepatocytes are known to produce huge amounts of the enzyme which is also used as a diagnostic tool for hepatic disfunction (Gomez et al., 2000; Kean et al., 1986; Santarpia et al., 2013). Our immunohistochemical results support that data (Fig. 5), showing a spread positivity in parenchymal hepatocytes. Furthermore, we also introduce an interesting novelty: the positivity for BChE of specific cell types: cholangiocytes, the epithelial cells of the bile duct, which contribute to bile secretion and hepatocyte survival by transporting bile acids (Banales et al., 2019).BChE levels are quantitative increased in plasma and liver of HFD treated mouse compared to normal chow diet counterparts (Kean et al., 1986; Siskova et al., 2016) as shown by our immunohistochemical data (Fig. 5 D,E,F). The panel in figure 5 also show a higher magnification of ducts in both diet conditions. Normal diet animals show specific BChE staining for cholangiocytes lining the duct surrounded by negative parenchymal cells (Fig.5C), a novel information not reported in literature according to our knowledge. Moreover, we visually identified and described in HFD steatotic liver a largely diffused positivity in parenchymal areas (Fig.5F), in accordance with its quantitative increase described in literature.

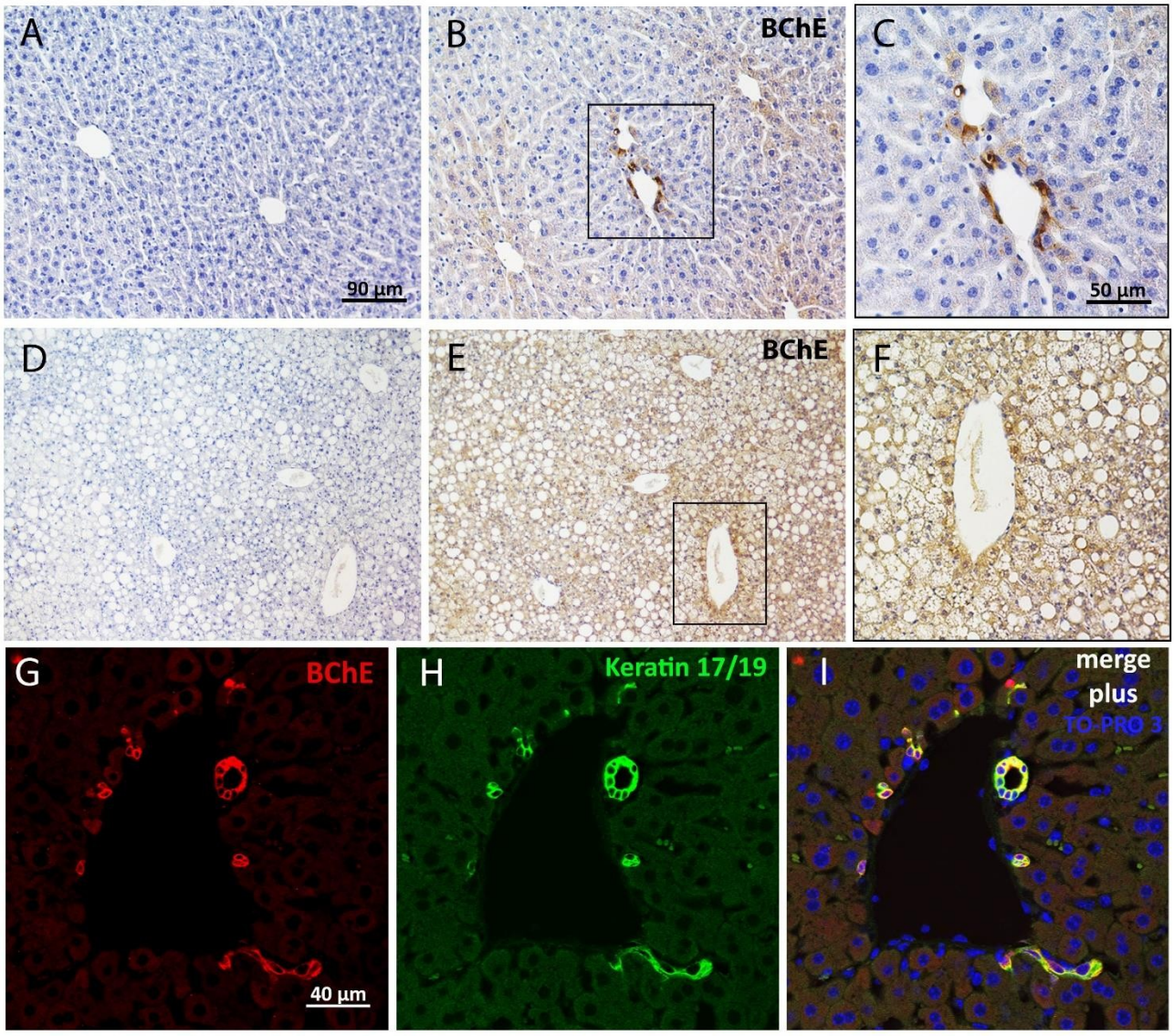

Figure 5| *Distribution of BChE in the mouse Liver.* A, D) Negative controls; B) Distribution of BChE in the liver of a normal diet fed mouse; E) Distribution of BChE in the liver of a high fat diet mouse. G) BChE positive cells surrounding a hepatic duct of a normal diet fed mouse; H) in green Keratin 17/19 distribution in cholangiocytes contouring a hepatic biliary duct; I) merge. Yellow staining indicates the correspondence of the cytotype expressing both antibodies: BChE and Keratin 17/19. In blu To-PRO 3 stains cell nuclei (dilution 1:3000). Scale bar: 90 µm A, B, D, E; 50µm C, F; 40µm G, H, I.

5.1.2 Expression of BChE in the liver under normal and high fat diet conditions

Levels of BChE are higher in HFD condition in the mouse liver (Chen et al., 2017a; K. Šišková, 2015). To validate this data we performed a Western blotting analysis on liver lysates of 3 normal chow-diet fed wild type C57BL/6J mice and 3 HFD- fed wild type C57BL/6J mice. In figure 6, the band corresponding to the protein at 75 kD is visible. BChE content was significantly higher in liver samples from HFD mice compared to chow diet (Fig.6, n=3, $p<0.05$).

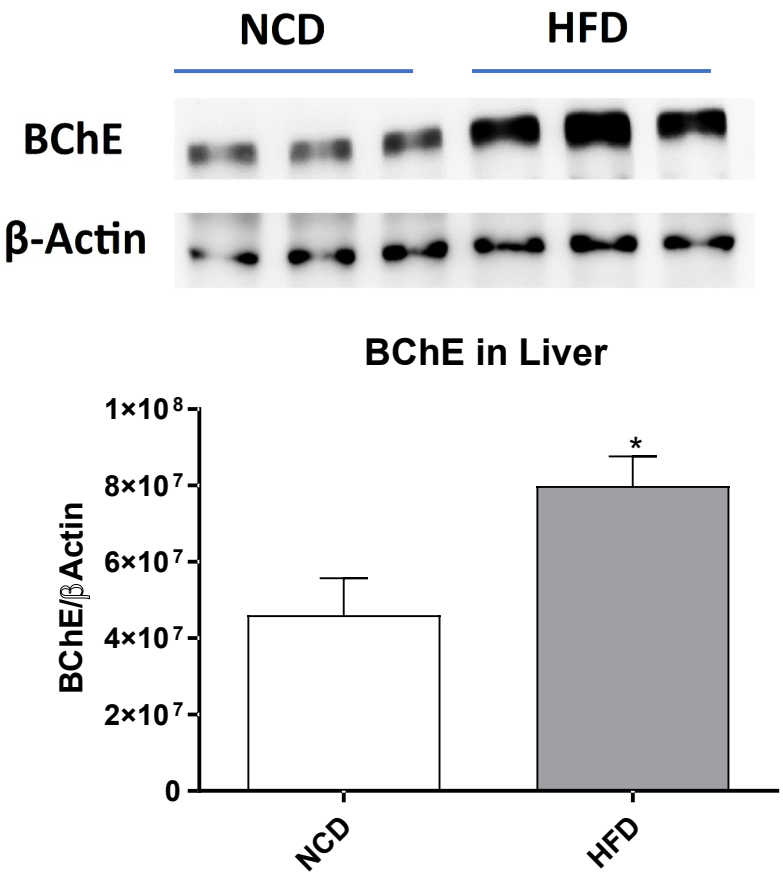

Figure 6| *Representative Image of Western Blot analysis for BChE protein (75 kD) in liver lysates of wild type C57BL/6J mice.* β-Actin served as a housekeeping. (NCD: Normal chow diet; HFD: High fat diet. N=3 for each group).

5.1.3 Distribution of BChE in the central nervous system

The presence of BChE in the central nervous system (CNS) is largely spread since it is an enzyme important for the regulation of the cholinergic system (Darvesh and Hopkins, 2003). Due to its correlation with the neurotransmitter AChE and its hydrolytic action towards that molecule (Silver, 1974), BChE is essential for the interruption of ACh neurotransmission. Many studies refer to BChE distribution into the CNS and a lot of nuclei are described (Reid et al., 2013). Here we take into consideration three of the most expressing nuclei in the brain and one in the brainstem.

In figure 7, BChE expression in a coronal section from the thalamus at the level of Dorsal Lateral Geniculate (DLG) nucleus. This thalamic nucleus is located under the Dentate Gyrus of the Hippocampus, not so far from the third Ventricle (3V) and it is densely populated with BChE-positive neurons. In the panel on the left (Fig. 7B) a higher magnification of the soma of some BChE-positive neurons.

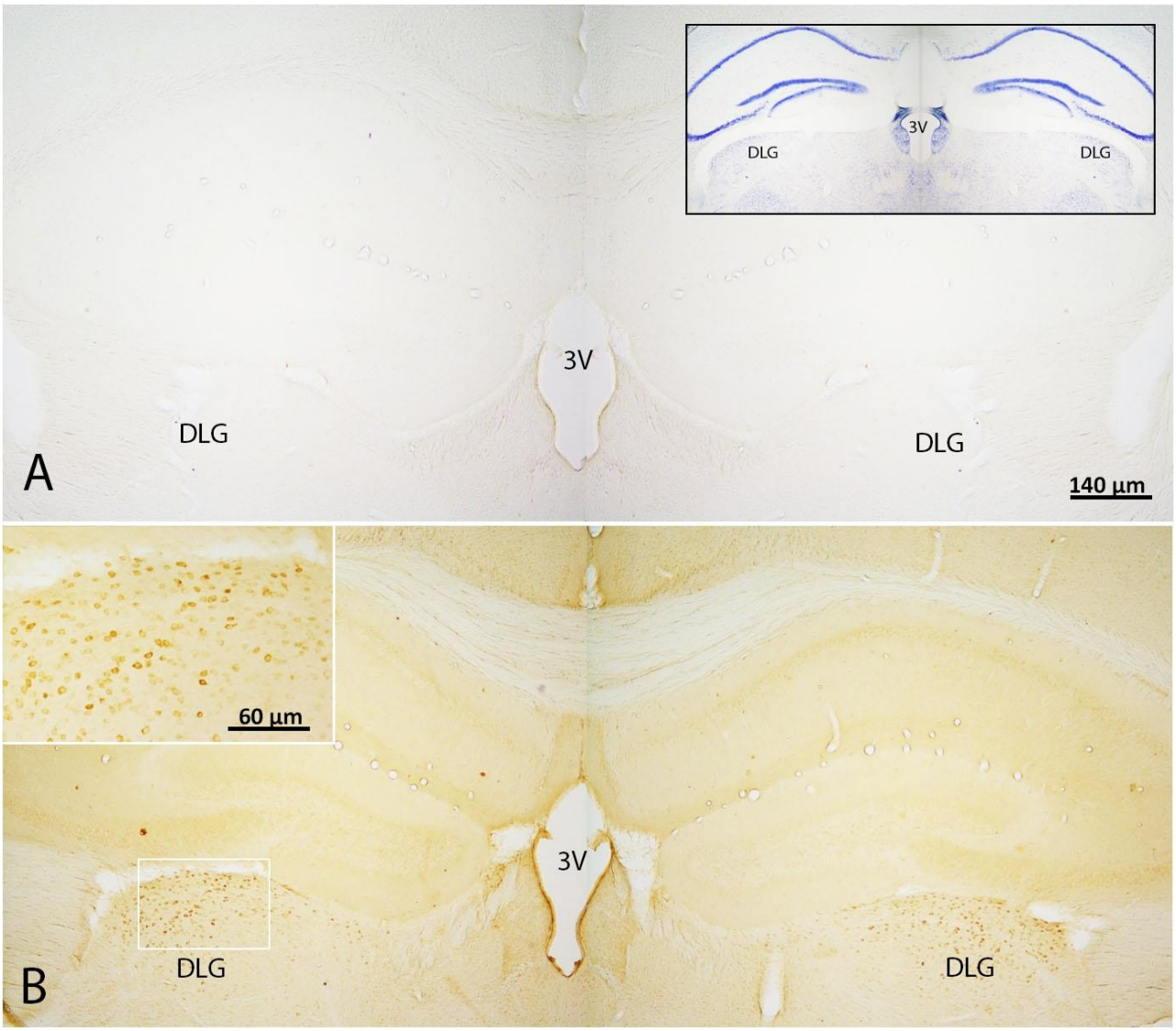

Figure 7| *Distribution of BChE in the mouse Thalamus.* A) Negative control and Nissl staining; B) Distribution of BChE in Dorsal Lateral Geniculate nucleus of the Thalamus (DLG) 3V- Third Ventricle, DLG- Dorsal Lateral Geniculate nucleus. Scale bar: 140 µm A and B, 60 µm insert in B.

BChE positive cells are also present in the medial habenula (Fig. 8).

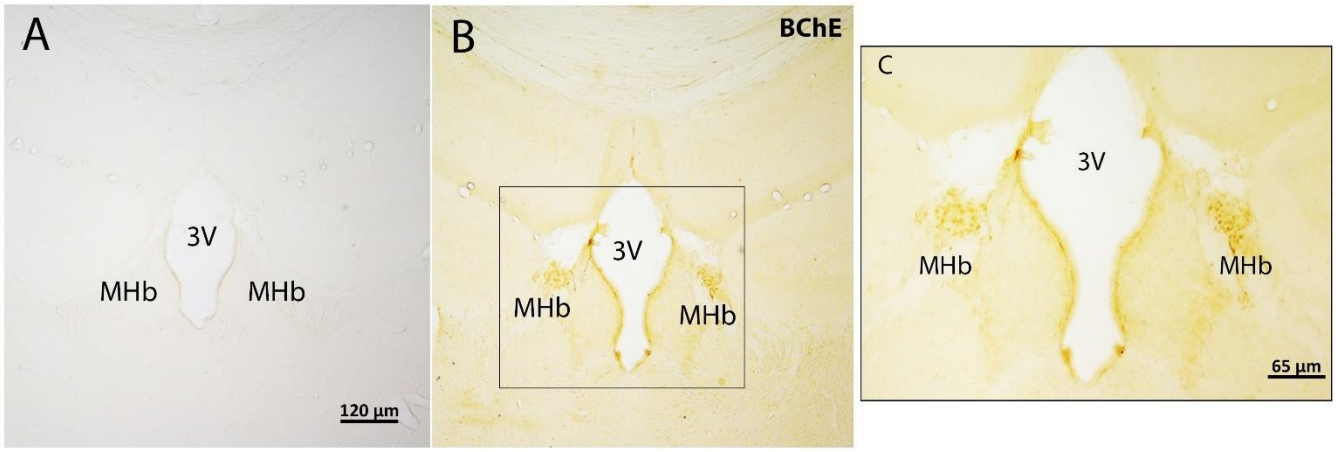

Figure 8| *Distribution of BChE in the mouse Medial Habenula.* A) Negative control; B) Distribution of BChE in Medial habenula (MHb). 3V- Third Ventricle, MHb - Medial habenula. Scale bar: 120 µm A, B; 65 µm C.

A densely positive populated area is also the Nucleus of Reuniens (RE) (Fig. 9)

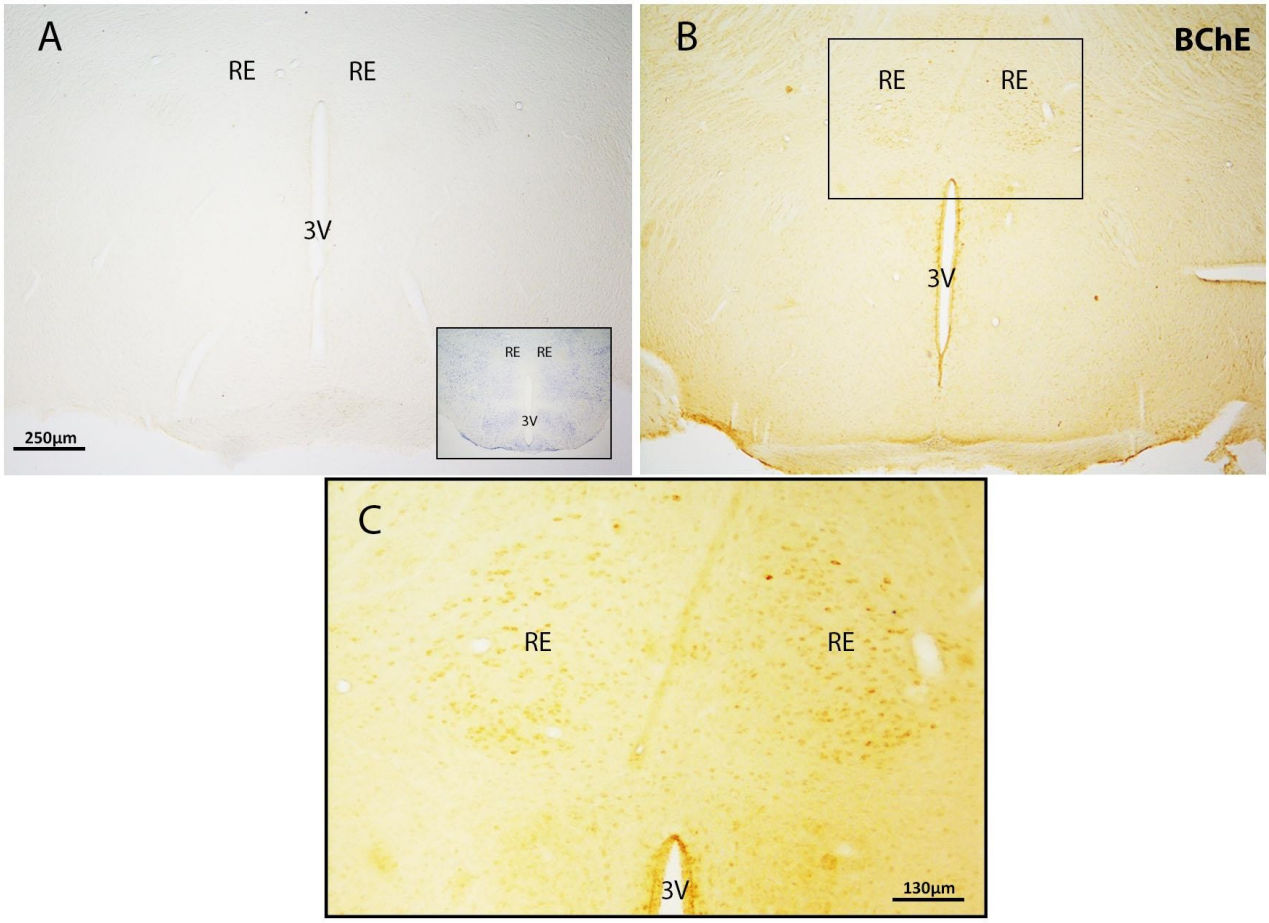

Figure 9| *Distribution of BChE in the mouse Hypothalamus.* A) Negative control; B) Distribution of BChE in Nucleus of Reuniens C) Enlargement of panel B. 3V- Third Ventricle, RE – Nucleus of Reuniens. Scale bar: 250 µm A, B; 130 µm C.

BChE-positive cells are present also in brainstem sections in the Dorsal motor Nucleus of the Vagus (DMX) (Fig. 10), as previously reported (Reid et al., 2013).

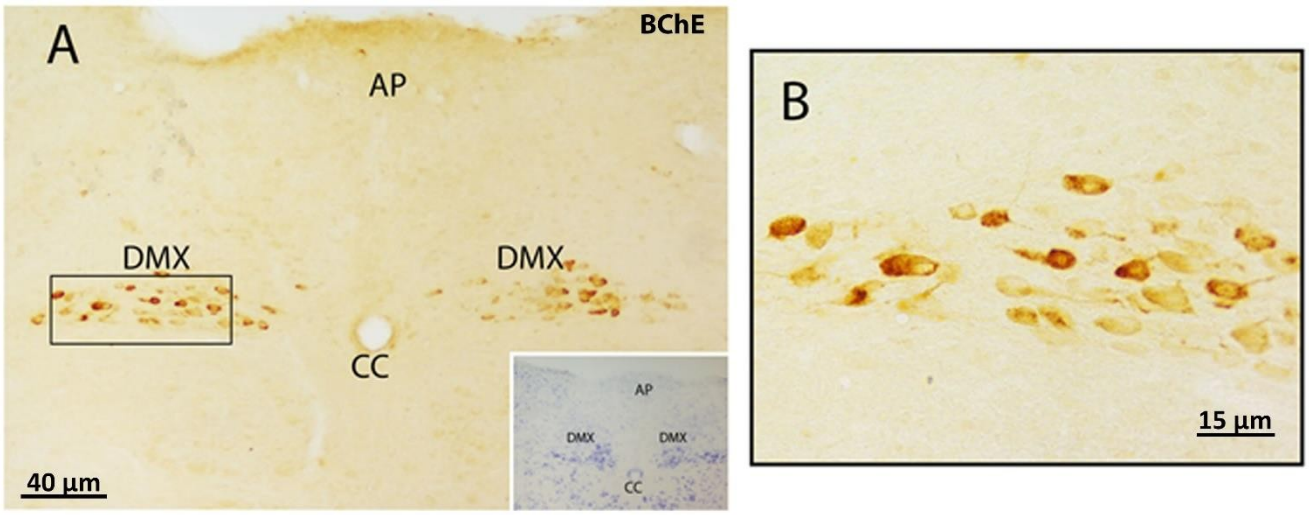

Figure 10| *Distribution of BChE in the mouse brainstem.* A) Distribution of BChE positive cells in a brainstem coronal section and Nissl staining. B) Higher magnification of positive-stained cells into DMX nucleus. CC- Central Canal, AP- Area Postrema, DMX- Dorsal motor nucleus of the vagus nerve. Scale bar: µm A; µm B. Scale bar: 40 µm A; 15 µm B.

5.1.4 Distribution of BChE along the gastro-intestinal tract

5.1.4.1 Distribution of BChE in the salivary glands

The oral cavity is underlined by a mucosal membrane and it is always moistened by the saliva secreted by the associated major and minor salivary glands. (Amano et al., 2012) These glands are composed of three pairs of glandular organs: parotid, sublingual and submandibular. In the mouse, sublingual and submandibular glands are associated and incapsulated with a common fascia (Amano et al., 2012). They secrete saliva into the oral cavity through a series of ducts in the ductal system allowing the saliva to absolve its major functions: digestive, antibacterial, buffering, lubricant and water-balance in the oral cavity itself. Salivary glands are anatomically composed by glandular acini (or alveoli) and ducts. Depending on the particular salivary gland, these acini are lined by variable combination of mucous and serous epithelial cells surrounded by myoepithelial cells, located between the basal plasma membrane and the acinar basement membrane (CL Maruyama, 2018). Figure 11 shows a section of mouse sublingual gland. In panel B, BChE positive ductal epithelial cells of the gland. This data is more appreciable from panel C and D where it is possible to find two major enlargements of the ductal component of the gland.

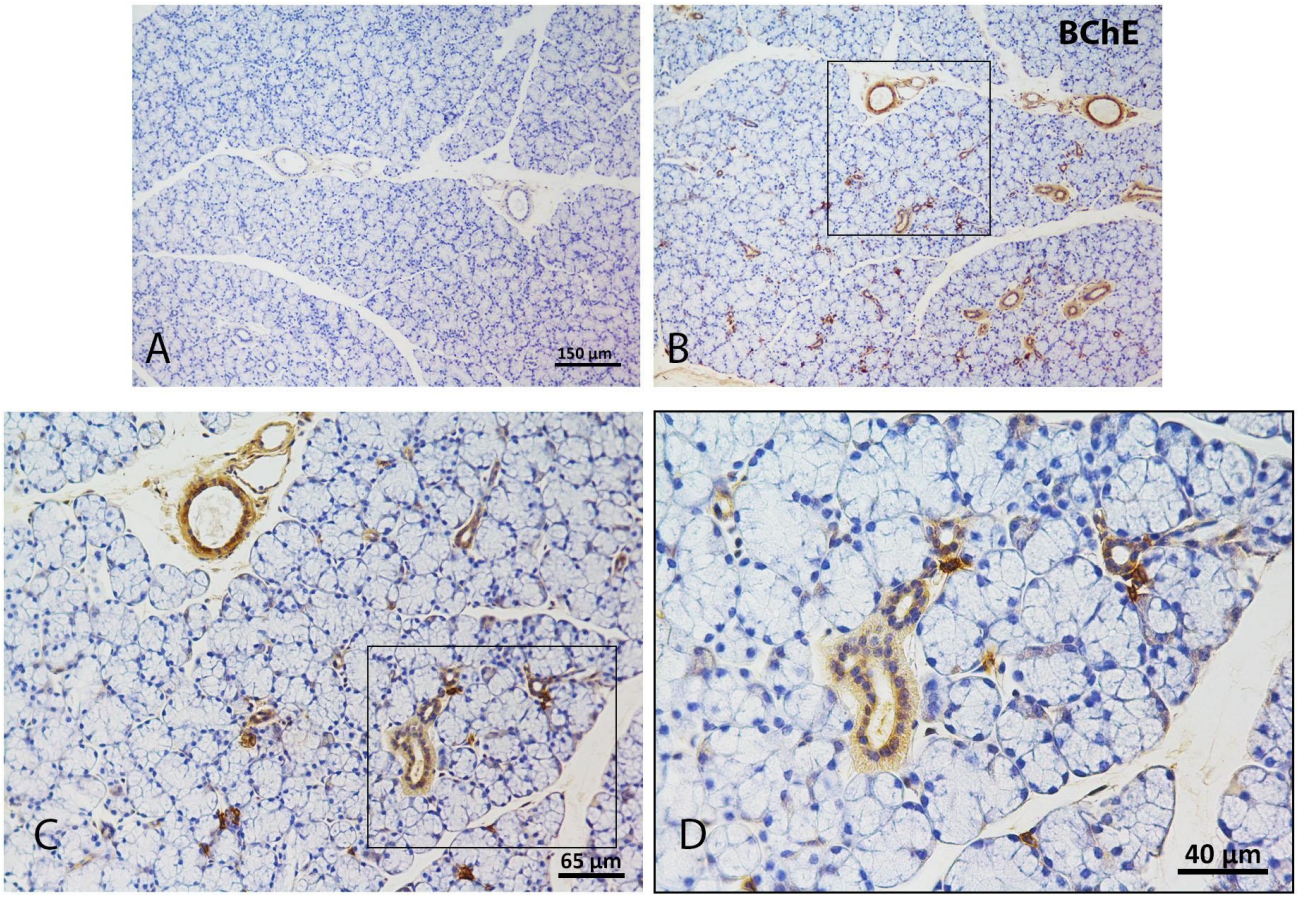

Figure 11| *Distribution of BChE in the mouse salivary glands*. A) Negative control; B) Distribution of BChE in ductal portion of mouse salivary gland; C, D) higher magnification. Scale bar:150 µm A, B; 65 µm C; 40 µm D.

5.1.4.2 Distribution of BChE in the esophagus

Esophagus is one of the upper gastrointestinal organs. In rodents the esophageal wall is organized as a muscular tube which surrounds a central lumen, composed of multiple layers. The inner luminal surface of rodent esophagus is composed by keratinized stratified squamous epithelium 3-5 cells thick (thickness of that layer is diet-dependent; fasting animals often increased keratin layers and show adherent bacterial colonies).

Figure 12 show BChE distribution in an esophageal-3µm section. In B, BChE positive cells are localized in the inner keratinized squamous epithelium. Interestingly, also the luminal content seems BChE-positive suggesting a possible secreting function of the esophageal epithelial cells. At higher magnification, in the panel above scattered positive cells in the muscularis mucosa are present, which strength the already known concept of the presence of BChE in rodents and human skeletal muscles (Jbilo et al., 1994).

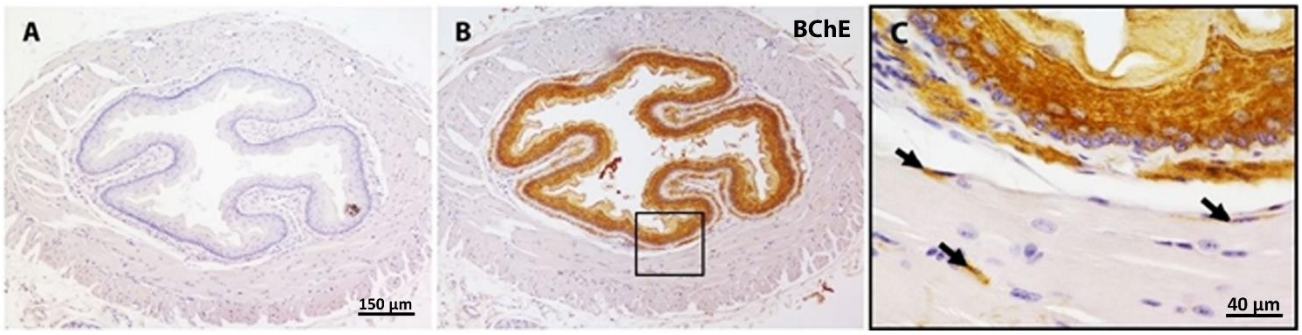

Figure 12| *Distribution of BChE in the mouse esophagus*. A) Negative control; B) Distribution of BChE in esophageal epithelium; C) higher magnification of B. Black arrows indicate BChE positive cells in esophageal muscularis mucosa. Scale bar: 150 µm A, B;40 µm C.

5.1.4.3 Distribution of BChE in the stomach

The stomach is a dilated portion of the gastrointestinal tract responsible for storing and processing food into chyme and then delivering measured amounts to the duodenal portion of the small intestine. In the mouse, the stomach is grossly divided into two distinct regions that are visible even from the serosal aspect: the forestomach, or non-glandular stomach and the glandular stomach. The forestomach is lined by keratinizing squamous mucosa (Luciano and Reale, 1992), which accounts for approximately two-thirds of the organ and lies to the left of the limiting ridge (the visible, slightly raised division between the non-glandular and glandular regions which resembles the human Z-line), encompassing the entrance of the lower esophagus. The mouse glandular stomach is histologically divided into three anatomic regions: the small cardia adjacent to the limiting ridge; the fundus, where the mucosa is folded with rugae; and the antrum, which has relatively smooth mucosa.

In figure 13, sections of the mouse glandular stomach at the level of the fundic area, the largest region, are visible. The mucosa is occupied mostly by gastric glands, which empty into short gastric pits (we can distinguish the pits located in the lower part of the left area of figure A and B). In B, numerous BChE positive cells are present interspersed into glands structures.

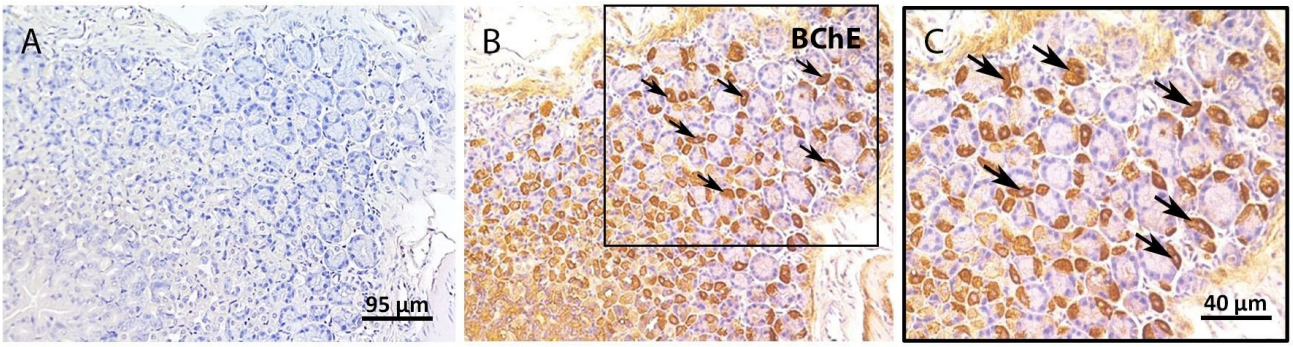

Figure 13| *Distribution of BChE in the mouse stomach fundus*. A) Negative control; B) Distribution of BChE; C) Higher magnification of a glands-rich area with many positive cells. Black arrows indicate some positive cells in gastric glands structures. Scale bar: 95 µm A, B;40 µm C.

These cells are pyramidal-shape cell types, with a peripherally located nucleus. The positivity is clear and specific since in a glandular structure only few cells are positive-stained and are localized immediately next to negative cytotypes. In C at higher magnification gland structures are visible, with positive cell types next to negative ones, and some entirely negative fundic glands. Analyzing various stomach sections, we sought to define if there were variations in BChE-positive cells distribution in different areas composing mouse glandular stomach starting from the small cardia region, which lies in apposition with the distal portion of the squamous forestomach adjacent to the limiting ridge (Fig. 14 A, B), passing by the larger fundic region (Fig. 14 C,D,E,F), until the distal pyloric region (Fig. 14 G,H) which opens into the duodenum.

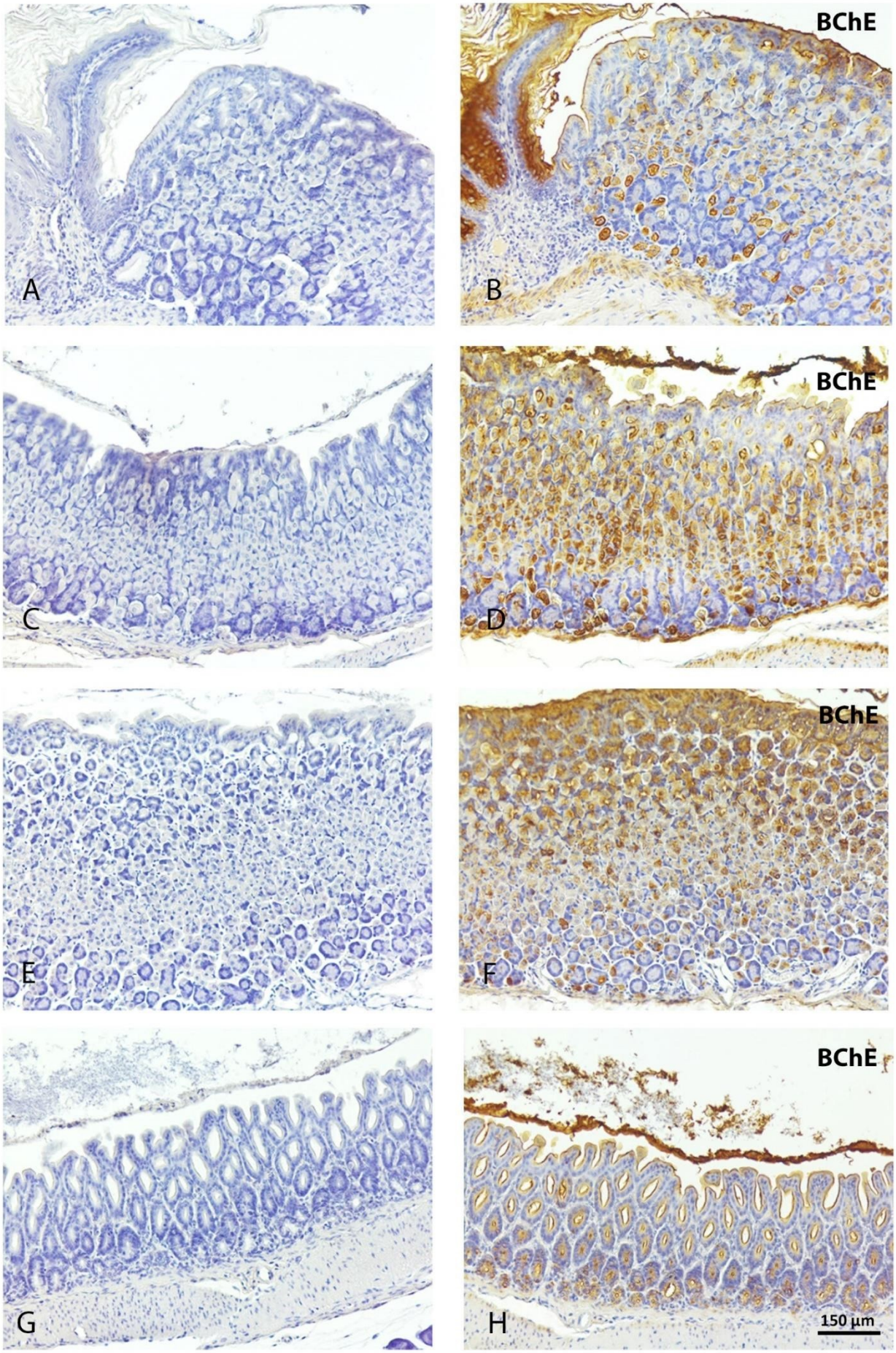
A
BChE
B
C
BChE
D
E
BChE
F
G
BChE
H
150 µm

Figure 14| *Distribution of BChE in different areas of the mouse glandular stomach*. Paraffin section of cardia (A, B, fundus (C, D, E, F), antrum (G, H), are shown in the panel. A, C, E, G) Negative control; B, D, F, H) Distribution of BChE in different portion of mouse stomach. Scale bar: 150 μm A-H.

The cardia region (B) is rich in BChE-positive cells, some of them intensely stained, situated in the lower part, adjacent to the submucosa, while others with a weaker staining in the middle- upper part, next to the lumen of the stomach. The higher number of BChE-expressing cells was noticed in the fundic region (D, F). Abundant marked cells are present in the whole mucosa with a prevalence for the lower-middle portion. In the upper part of picture F secreted, positive-stained material inside the glands located in the apical portion of the oxyntic mucosa is visible. The prevalence of positive cell types remarkably decreases in the antrum region, an area composed only of mucous cells, where cytotypes which are predominant in the glandular stomach (parietal cells and chief cells) are absent.

5.1.4.3.1 Characterization of BChE-expressing cytotypes in the gastric oxyntic mucosa: a double labelling study

To BChE-expressing cells, we performed double labelling studies with the principal marker of the glandular stomach cells: Parietal cells, chief cells, neuroendocrine cells, and ghrelin- producing cells. We used the H/K ATP-ase antibody to mark Parietal cells, since H/K-ATPase is a proton pump abundantly diffused in the cytoplasmatic tubulo-vesicles responsible for proton extrusion during acid secretion, the main task of Parietal cells (Arin et al., 2017) . Through an immunohistochemical staining we obtained a map of distribution of parietal cells in the gastric fundic mucosa of mouse. The distribution of that cell type (Fig.15 B) is abundant and largely diffused in the gastric oxyntic mucosa. A larger number of cells is clearly distributed in the middle portion of the glandular area, less scattered cells can be found at the base of the crypts. We performed a double labelling-fluorescent reaction using an anti-H/K ATP-ase antibody and an anti-BChE antibody (Fig.15 C, D, E). In red, BChE distribution (C) in a section of glandular stomach, in green H/K ATPase positive cell types (D), while in E is shown an overlay of the two channels. Colocalization of the two antibodies clearly appears from the yellow staining of the cytotypes in figure 15 E. Parietal- BChE positive cells are located in the lower-middle portion of the gastric oxyntic mucosa, mostly in the crypt area and are a subpopulation of Parietal cells.

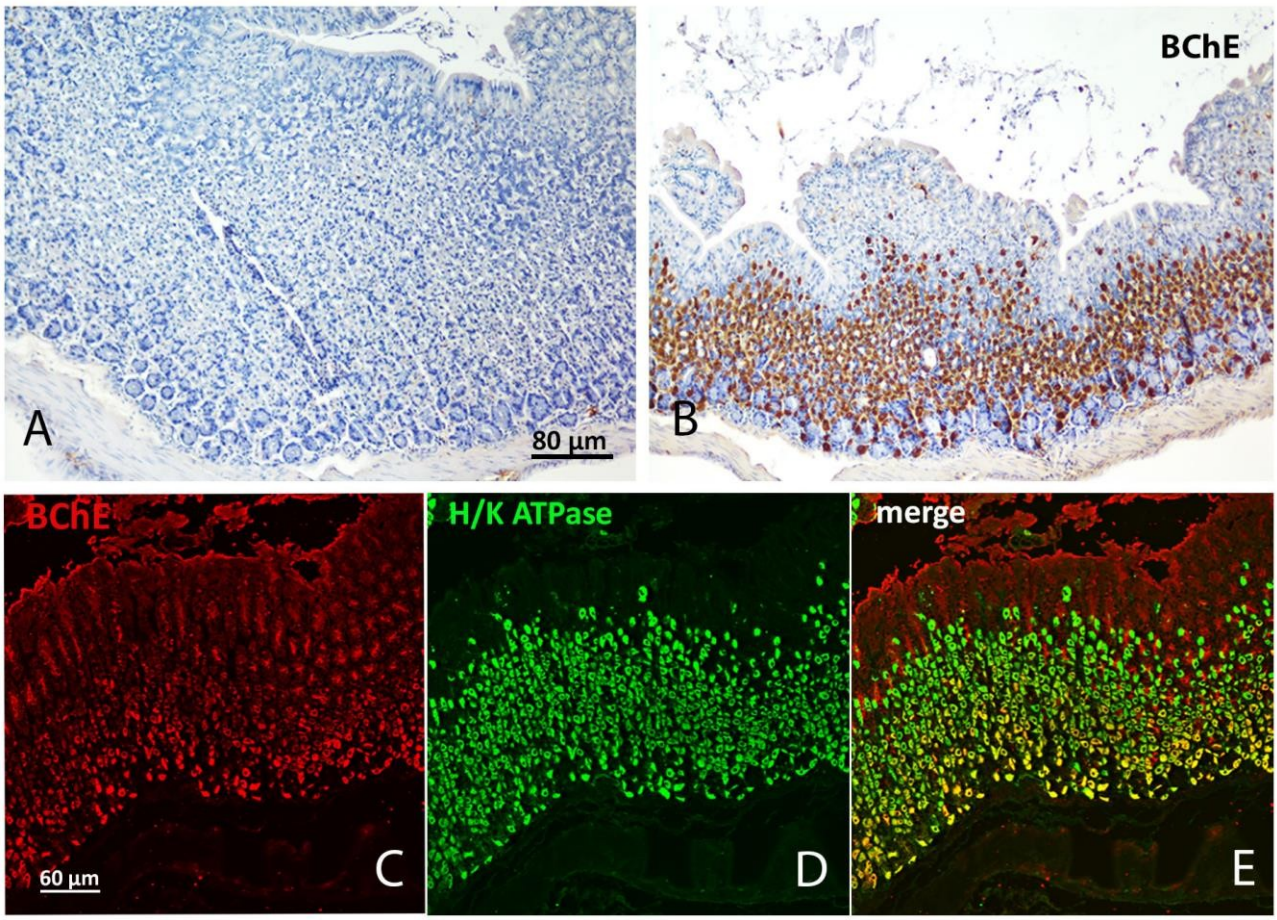

Figure 15| *Distribution of H/K ATPase in the stomach fundus of mouse.* A) Negative control; B) Distribution of H/K ATPase in mouse gastric oxyntic mucosa; C) in red positive staining for BChE; D) in green the distribution of parietal cells labelled with H/K-ATPase antibody; E) merge. Yellow staining show colocalization. Scale bar: 80 µm A, B; 60 µm C, D, E.

To confirm that BChE is expressed only in parietal cells and not by other cell types, we also examined the distribution of Gastric Intrinsic factor (GIF), considered the marker of mature rodent chief cells. Different from humans, where gastric intrinsic factor is the major secreted protein of the parietal cells, in mouse and rat stomach GIF is secreted by chief cells (Shao et al., 2000).

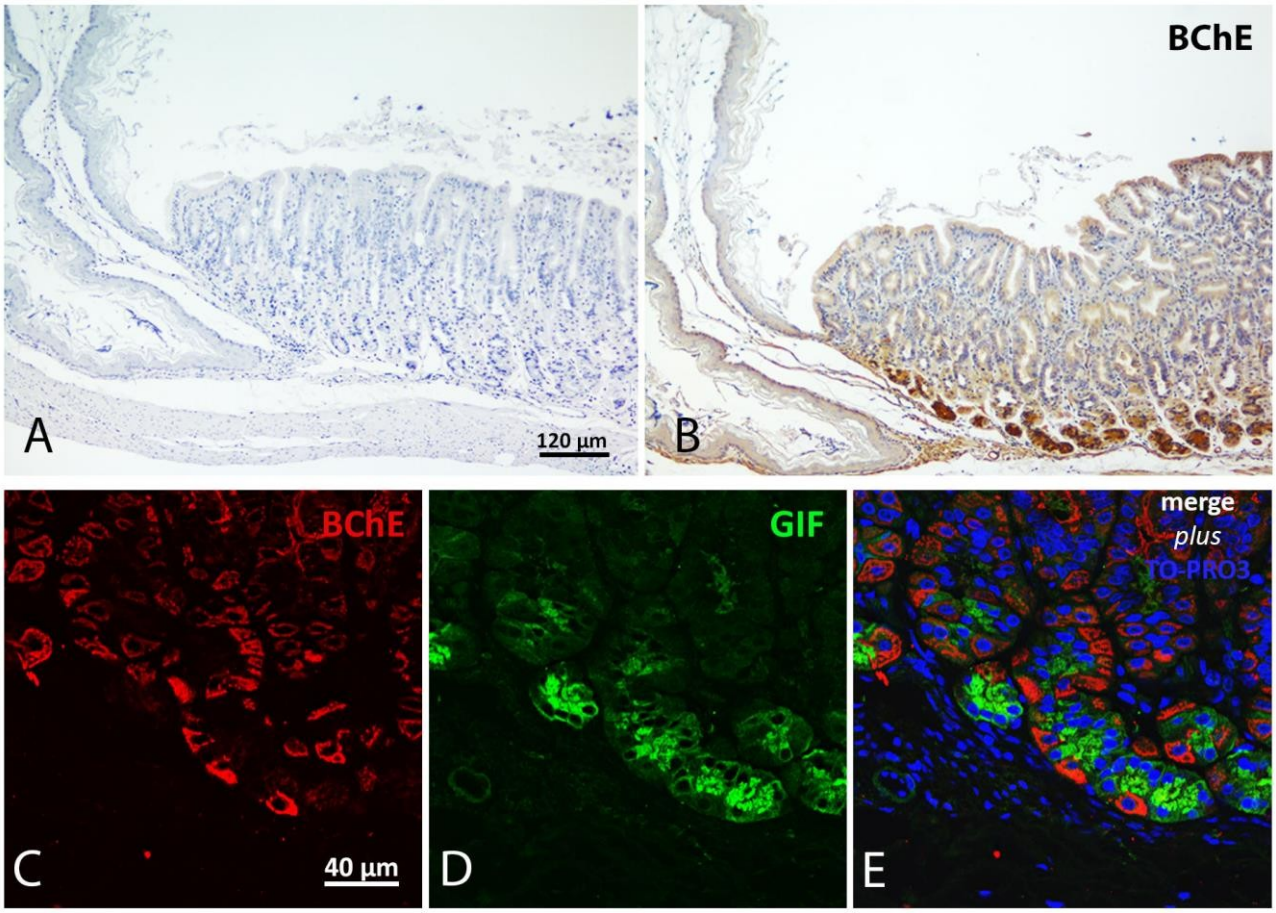

Figure 16| *Distribution of Gastric Intrinsic Factor (GIF) in the stomach fundus of mouse.* A) Negative control; B) Distribution of GIF in mouse gastric oxyntic mucosa; C) in red positive stained cells for BChE; D) in green the distribution of chief cells labelled with GIF antibody; E) merge. In blu To-PRO 3 stains cell nuclei (dilution 1:3000). Scale bar: 120 µm A, B; 40 µm C, D, E

Immunohistochemical staining for Gastric Intrinsic Factor in the glandular stomach shows a deeply different distribution compared to the one of BChE. GIF is expressed in chief cells at the bases of oxyntic glands (Fig.16 B). Double labelling confocal microscopy analysis performed with BChE antibody and GIF antibody was performed on stomach sections. In C BChE staining in red, in D the green staining of GIF positive chief cells. The overlay of the two channels is shown in figure 16 E, where no colocalization between the two staining was visible. BChE positive cells are not GIF positive cells.

In order to confirm our hypothesis of the parietal cells as the only BChE producer cytotype in glandular stomach, we analyzed also the neuroendocrine cell type using Chromogranin-A (Chr-A) antibody. The immunohistochemical staining obtained give us an idea of the distribution of that cytotype. Panel 17 A and B show neuroendocrine cells distribution. In B black arrows indicate the Chr-A-positive cells interspersed between negative cells. They are scattered cells, mostly localized

in the lower part of the glands, proximal to the submucosa. Comparing that data with the immunohistochemical staining for BChE we can already speculate that we are taking into consideration two different cell types: the morphology and the number of positive cells, are clearly not comparable. To confirm that hypothesis, we performed a double labelling reaction with BChE antibody (Fig. 17). Panel C shows in green BChE-positive cells. panel D red-Chr-A positive neuroendocrine cells, while in E the overlay of the two. Our hypothesis was confirmed: BChEpositive cells are not neuroendocrine cells.

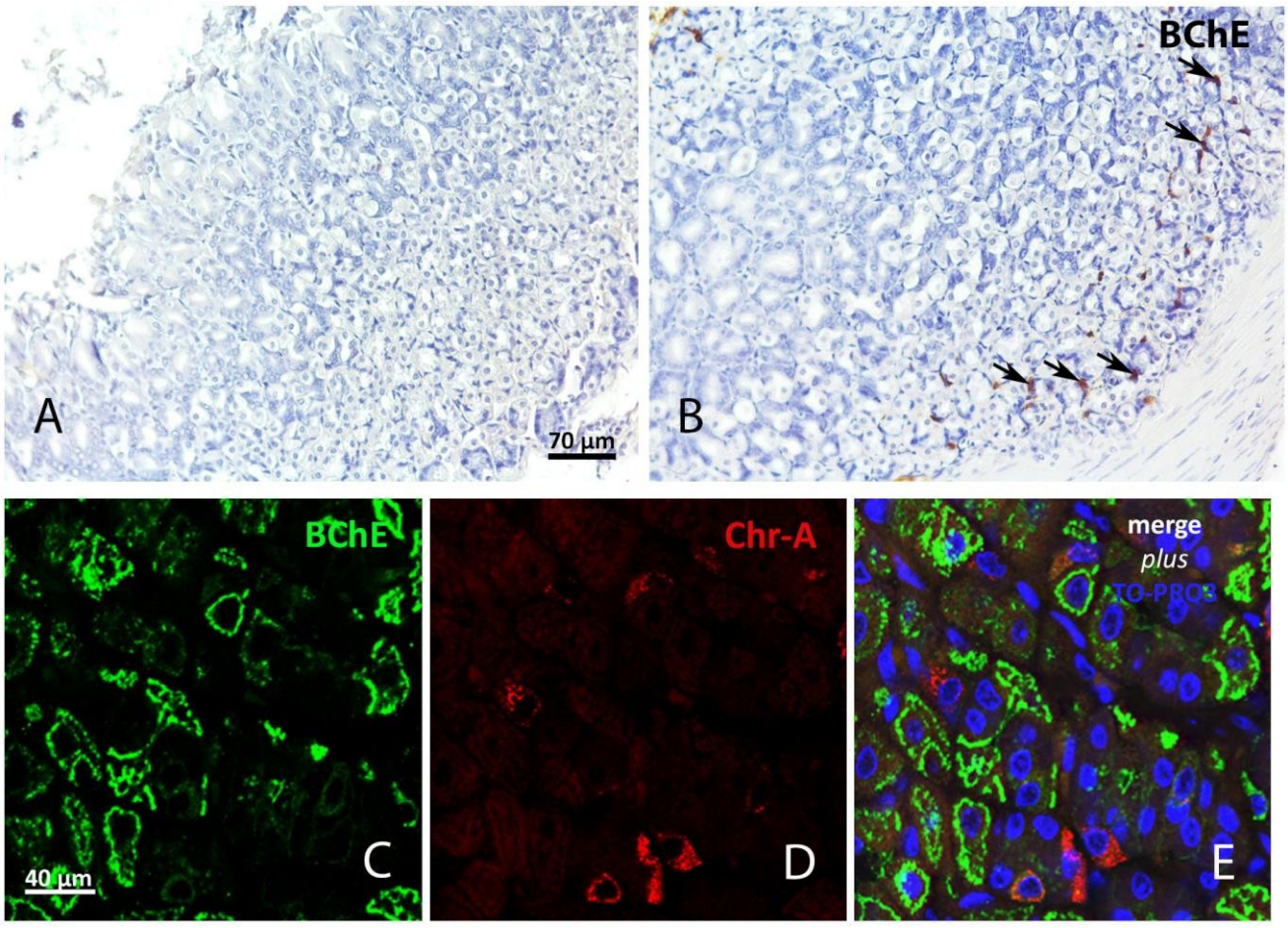

Figure 17| *Distribution of Chromogranin-A (Chr-A) in the stomach fundus of mouse.* A) Negative control; B) Distribution of Chr-A in mouse gastric oxyntic mucosa. Black arrows indicate positive cell types; C) in green positive staining for BChE; D) in red the distribution of neuroendocrine cells labelled with Chr-A antibody; E) merge. In blu To-PRO 3 stains cell nuclei (dilution 1:3000). Scale bar:70 µm A,B; 40 µm C,D,E

5.1.4.3.2 Distribution of BChE and ghrelin producing cells

Ghrelin is considered a gut-brain peptide and IT is abundantly produced from endocrine cells in the gastrointestinal mucosa. In the gastrointestinal tract, ghrelin cells are most abundant in the stomach and are localized in gastric mucosal layers (Ichiro Sakata, 2010).The distribution of ghrelin-producing cells in the gastrointestinal tract has been studied through immunohistochemical techniques and in situ hybridization (Date et al., 2000; Dornonville de la Cour et al., 2001; Sakata et al., 2002) In rodents, ghrelin-producing cells were observed in all regions of the gastrointestinal tract: gastric fundus, antrum, duodenum, ileum, cecum, and colon. Ghrelin-producing cells were most dense in the gastric fundus and were found in the mucosal layer but not in the myenteric plexus in all the examined regions. In the stomach, most of the ghrelin cells were observed in the glandular base, decreasing in amount as the gland extends toward the lumen (Ichiro Sakata, 2010). As it is possible to see from panel 18 B and C ghrelin cell type in stomach appears as round cells mainly observed from the glandular base to the body of the fundic glands.

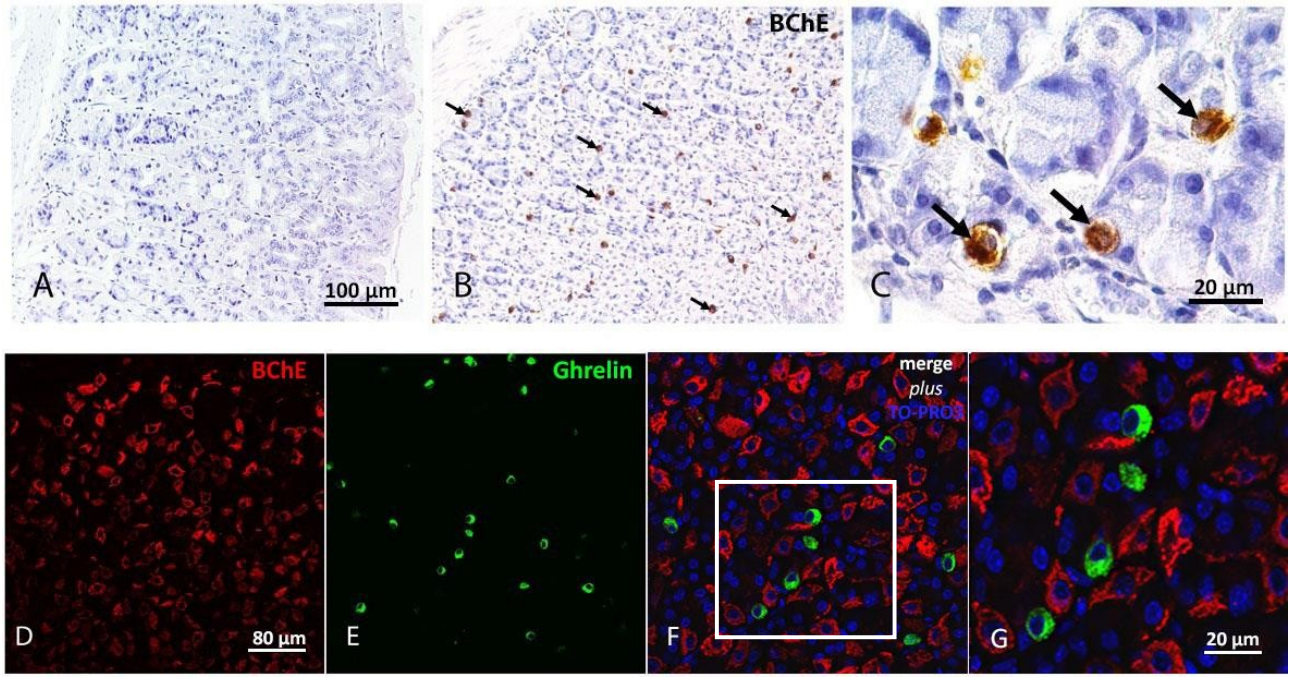

Figure 18| *Distribution of Ghrelin in the stomach fundus of mouse.* A) Negative control; B) Distribution of Ghrelin in mouse gastric oxyntic mucosa. C) Higher magnification of glandular structure with some positive stained cells. Black arrows indicate some of the positive cells in the stomach glandular parenchyma. D) in red positive staining for BChE; E) in green the distribution of Ghrelin producing cells; F) merge. G) Enlargement of an area of panel F. In blu To-Pro3 stains cells nuclei (dilution 1:3000). Scale bar:100 µm A,B; 20 µm C; 80 µm D,E,F; 20 µm G.

In order to understand if ghrelin producing cells are also BChE producing cells, we performed a double labelling analysis with the two antibodies of interest. Figure 18 D shows in red the positive

staining for BChE, giving a panoramic view of its distribution in the glandular stomach of mouse, while in E we can appreciate the staining of ghrelin producing cells in green. Figure 18 F represents the overlay of the two channels with small round green cells interspersed between red elongated cell types, BChE positive stained. In figure 18 G is shown an enlargement of panel C, where it is clearly shown, at higher magnification, the non-colocalization of the two cytotypes. Ghrelin-producing cells seem to be spatially related with BChE-positive cells but are clearly not the same cell types. We can state that this data again confirms and strengths our first observation of the parietal cells as the only gastric cytotype positive for BChE.

5.1.4.4 Distribution of BChE in the pancreas

Pancreas is a large gland composed by an exocrine and an endocrine portion. The exocrine pancreas is the largest portion, composed by acinar cells with pyramidal shape and amounts to 96–99% of total pancreatic volume. The endocrine pancreas composes the remaining 1–4% of the gland (Dolensek et al., 2015; Jurij Dolenšek, 2015). It comprises endocrine micro-organs called islets of Langerhans, which could be of circular to oval to highly irregular cross-sections and are composed of a few to several thousand endocrine cells. Beta cells make up 60–80% of islet's cells and are responsible for insulin secretion (Boland et al., 2017), Alpha cells secreting glucagon represent 10–20% of the total number of cells in mouse (Huang et al., 2011); Delta cells and PP cells releasing somatostatin and pancreatic polypeptide, respectively, are the least frequent cell type present less than 5% of cells in mouse (Briant et al., 2018; Zhao et al., 2020); Finally, the hormone ghrelin is released from epsilon cells which present less than 1 % of cells (Jo J, 2007; Kim A, 2009).

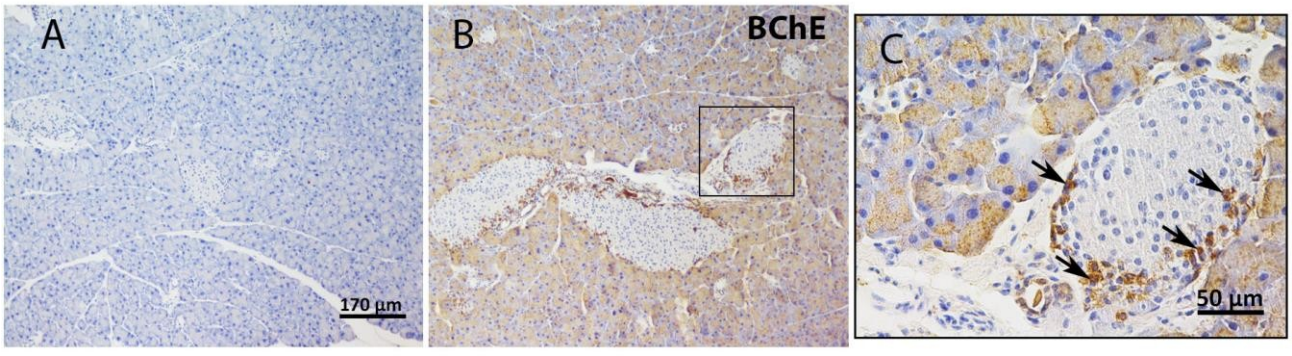

Figure 19| *Distribution of BChE in the mouse pancreas.* A) Negative control; B) Distribution of BChE in mouse pancreatic section; C) enlargement of an islet of Langherans where black arrows indicate BChE positive stained cells in the periphery. Scale bar:170 µm A, B; 50 µm C.

In figure 19, BChE immunohistochemical staining of a mouse pancreatic section. It is easily distinguishable the Langherans islet structure: oval shaped interspersed between the parenchymal

acinar cells of the endocrine portion of the organ. A more specific view of a Langherans islet is shown in the enlargement of the panel above (Fig.19B). Interestingly, our immunohistochemical data describes the presence of BChE in both the exocrine and the endocrine pancreas. Brown-dotted acinar cells of the exocrine pancreas can be found all around the Langherans islets of the endocrine portion of the gland, in which we can distinguish some positive peripheral cells surrounding the islet, possibly identifiable with alpha cells for their distribution (Jurij Dolenšek, 2015).

5.1.4.5 Distribution of BChE in the spleen

The spleen is a secondary lymphatic organ primarily devoted to immune surveillance of the blood. Besides its function in clearing particulate and other antigens, micro-organisms and aged red cells from the blood, the spleen is involved in the final steps of B cell maturation in mouse. The organ is composed of two portions called withe pulp and red pulp with the dominance of the white pulp (Moon et al., 2008).

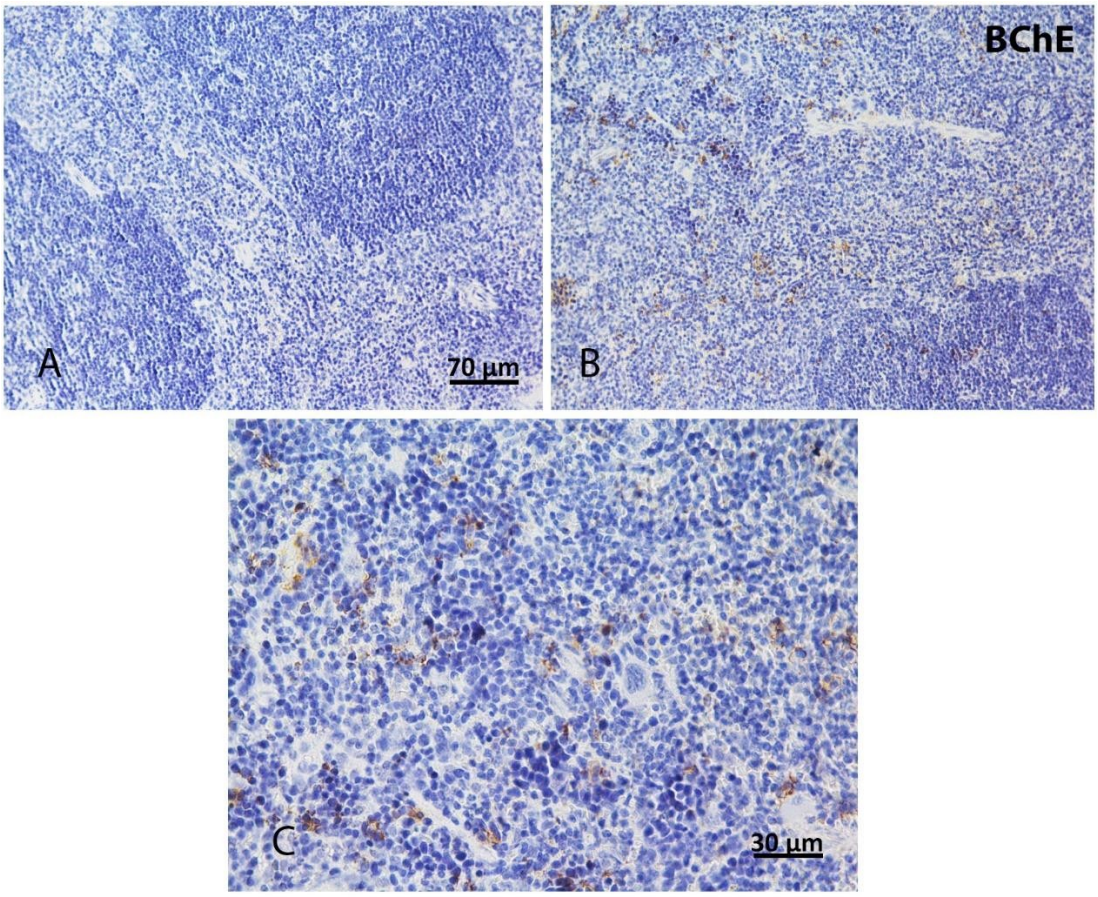

Figure 20| *Distribution of BChE in the mouse spleen.* A) Negative control; B) Distribution of BChE in a mouse spleen section with an enlargement at higher magnification C, of some positive-stained cells. Scale bar: 70 µm A, B; 30 µm C.

In figure 20, BChE positive cells are present. These cells are interspersed between red and white pulp of the organ and their heterogenous distribution and shape are possibly suggestive of macrophages.

5.1.4.6 Distribution of BChE in the small intestine

The small intestine is the longest portion of the gastrointestinal tract and it is responsible for the absorption of nutrients. It begins at the pyloric sphincter and continuous distally to the ileocecal valve. It is divided into three regions: duodenum, jejunum and ileum. Figure 21 shows a section of mouse duodenum. In A we can see negative control without primary antibody application, in picture B the positivity for the anti-BChE antibody. Positive staining is localized in particular structures which are characteristic of that intestinal portion and which are distinguishable from submucosa normal structure: the Brunner's Glands (B).

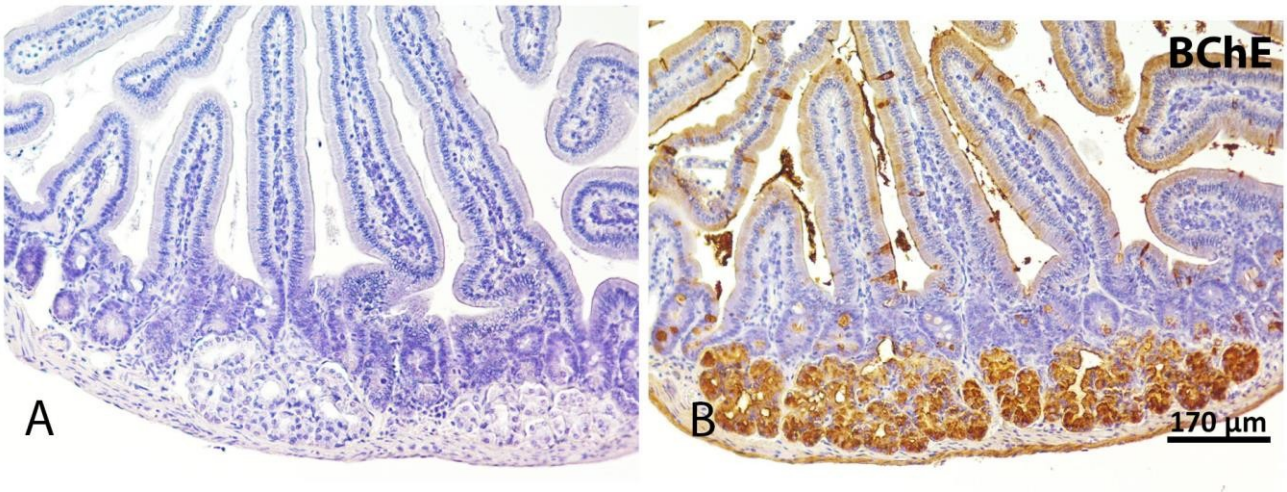

Figure 21 | *Distribution of BChE in the mouse duodenum.* A) Negative control; B) Distribution of BChE in the mouse duodenum. Scale bar: 170 µm A, B.

In jejunal portion of small intestine, Brunner's glands are no more present. Intestinal crypts are located at the base of the villi and represent simple and branched glands. Villous epithelium is contiguous with crypt epithelium, which is composed of surface absorptive cells (enterocytes), goblet cells, stem cells, Paneth cells and enteroendocrine cells. At this level, we detected specific positivity for BChE in structures located at the base of the villi. Figure 22 shows a negative control of a mouse jejunum paraffin section (Fig. 22A), followed by positive-stained cells in intestinal crypts (Fig. 22B) and some scattered positive cytotypes along the villus. Submucosa and muscularis regions which are located immediately above the crypts, shows no BChE-positive cells.

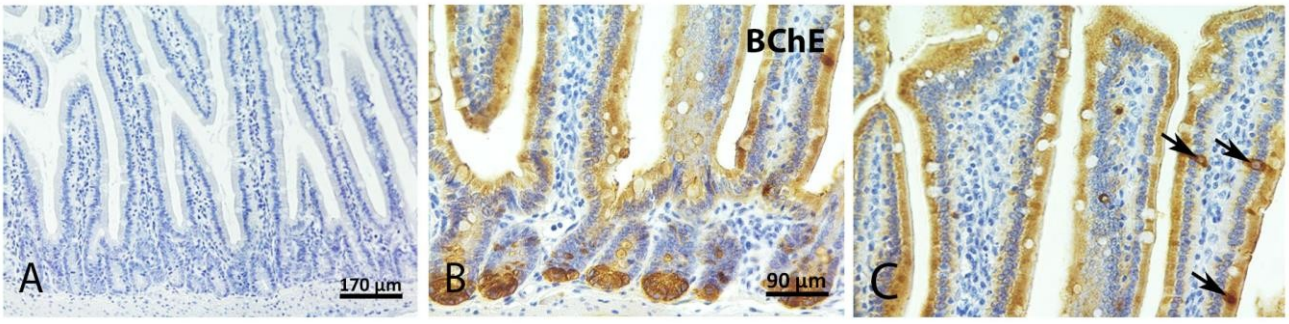

Figure 22| *Distribution of BChE in the mouse jejunum*. A) Negative control; B) Distribution of BChE in mouse intestinal crypts; C) Black arrows indicate positive staining of isolated cells along the villus. Scale bar: 170 µm A; 90 µm B,C .

5.1.4.6.1 Characterization of BChE responsive cytotypes in the intestinal crypts: a double labelling study

To identify and characterize the cytotype expressing BChE in mouse small intestine we performed a double labelling study with two specific markers: Lysozime and Olfactomedin 4 (Olmf4).

Once stated that the distribution of BChE positive cells was mostly linked to the crypt areas of small intestine, Lysozime, a marker of Paneth cells (Bel et al., 2017), was the first antibody to be tested. Paneth cells are known to reside at the base of the crypts of Lieberkühn (Sato et al., 2011), site of proliferative niche where reside ISCs which play crucial roles in maintaining intestinal homeostasis and mucosal immunity representing one of the four major epithelial lineages in the mouse small Intestine. Our immunohistochemical data, firstly show the distribution of Paneth cells at the crypt base in figure 23 A-C. In addition, performing a double labelling reaction with BChE and Lysozime antibodies (a marker of Paneth cells) in a mouse intestinal section (Fig. 23 D-I), we could state that BChE positive cells are also Lysozime positive cells (Fig 23 F and I, yellow staining). Figure 23 D shows some red stained cells positive for BChE antibody. In 23E, the distribution of Lysozime positive green cells is shown, while in 23 F is possible to appreciate the overlay of the two channels showing yellow cytotypes positive for both BChE and Lysozime antibodies. Moreover, it is possible to notice some scattered green positive cells composing the crypt which were not stained by BChE but marked with Lysozime. In conclusion, a subpopulation of Paneth cells located in cryptal areas of the mouse small intestine produce BChE. That data is also confirmed by the enlargements of figure 23 G-I. Here is possible to appreciate at higher magnification a crypt structure positive for BChE antibody in red (G), for Lysozime antibody in green (H), while figure 23 I shows with the overlay of the two channels an intestinal crypt enlargement with some yellow cells: Paneth cells producing BChE. Moreover, it is possible to see green-stained Paneth cells not positive for BChE and some red material into the

crypt which probably stands for BChE which has been secreted into the internal part of the crypt and ready to be reversed into the intestinal luminal content.

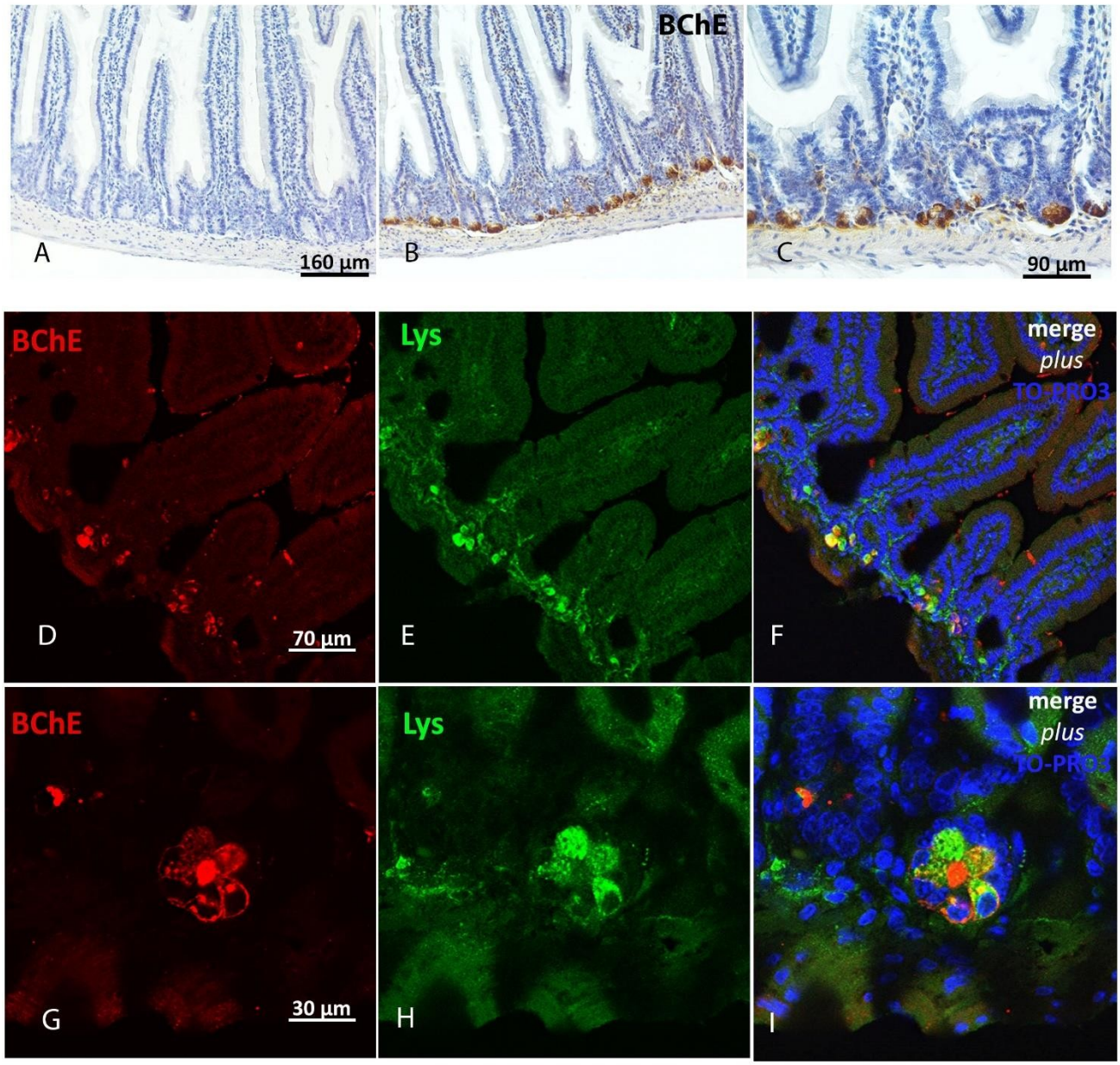

Figure 23| *Distribution of Lysozime in the mouse duodenum.* A) Negative control; B) Immunohistochemical staining for Lysozime in mouse duodenum; C) Higher magnification of a crypt with Lysozime positive cells. D) in red BChE positive cells; E) in green Lysozyme positive cells; F) merge. I) higher magnification of an intestinal crypt. In blu nuclear staining with ToPro-3 (dilution 1:3000), red BChE, green Lysozime. In the center some secreted BChE in red. Scale bar: 160 µm A,B; 90 µm B; 70 µm D,E,F; 30 µm G,H,I.

Olmf4 is a marker of intestinal stem cells (van der Flier et al., 2009). Since our immunohistochemical data localize BChE in intestinal crypt regions, and intestinal crypts are the location of the proliferative niche in large intestine (Sato et al., 2011), we performed a double labelling reaction to evaluate if BChE positive cells are stem cells. The panel in figure 24 shows the immunohistochemical

and immunofluorescent staining for Olmf4 antibody. Immunohistochemistry highlighted that its localization is at the base of the cryptal area of duodenal section. Moreover, immunofluorescence reveals red BChE-positive stained cells distribution in crypt, and green Olmf4 positive cells at the same time. The overlay of both channels in 24 F shows that they are not the same cytotype of Olmf4 expressing cells, but there is a spatial correlation between the two.

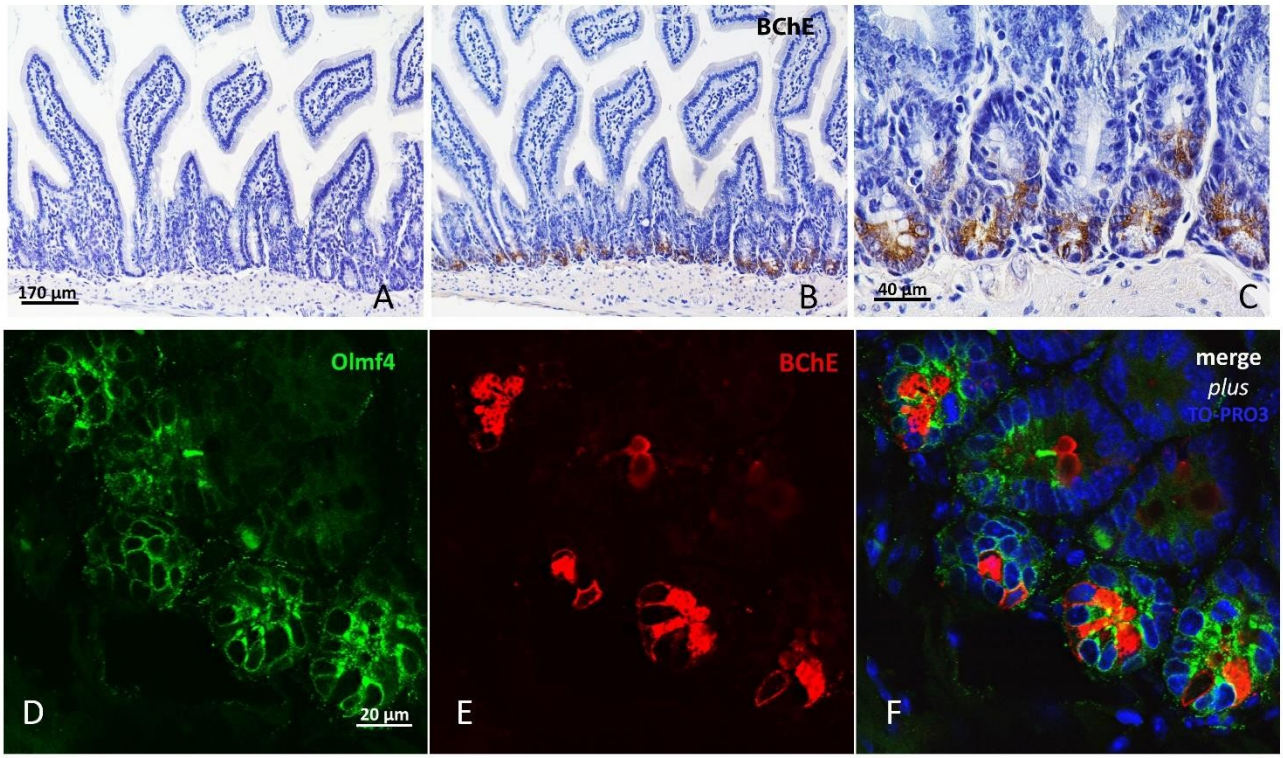

Figure 24| *Distribution of Olmf4 in the mouse duodenum* A) Negative control; B) distribution of Olmf4 in mouse duodenum; C) Higher magnification of a crypt with Olmf4 positive cells. D) Intestinal crypt of a crypt with Olmf4 positive cells in green E) In red BChE positive cells; F) merge. In blu nuclear staining with ToPro-3 (dilution 1:3000), red BChE, green Olmf4. Scale bar: 170 µm A, B; 40 µm C; 20 µm D, E, F.

5.1.4.7 Distribution of BChE in the colon

The large intestine of the mouse or large bowel is the last part of the digestive system. Its functions are to absorb water from the remaining indigestible food matter and then pass the useless waste materials from the body. It can be divided into three segments: the proximal colon originating from the ileocecal junction, the mid-colon, and the distal colon. Each segment represents approximately one-third of the total colon length. Mouse large intestine exhibits a thick mucosa with deep crypts but has no villi. The epithelium is composed by columnar absorptive cells, with a striated border,

many goblet cells, endocrine cells and basal multipotent stem cells, but no Paneth cells (Freeling and Rezvani, 2016). Our immunohistochemical data (Fig. 25) show the immunohistochemical distribution of BChE in a section of mouse large intestine. Numerous goblet cells and enterocytes are positive stained (Fig. 25 B and C). The enterocytes in the mucosa contain digestive enzymes such as peptidases, sucrases, maltase, intestinal lipases which could be responsible for the dotted staining into positive cells of panel B and C. In panel C, some positive-stained material into the intestinal lumen is present, while the underlying submucosa layer is clearly negative for BChE.

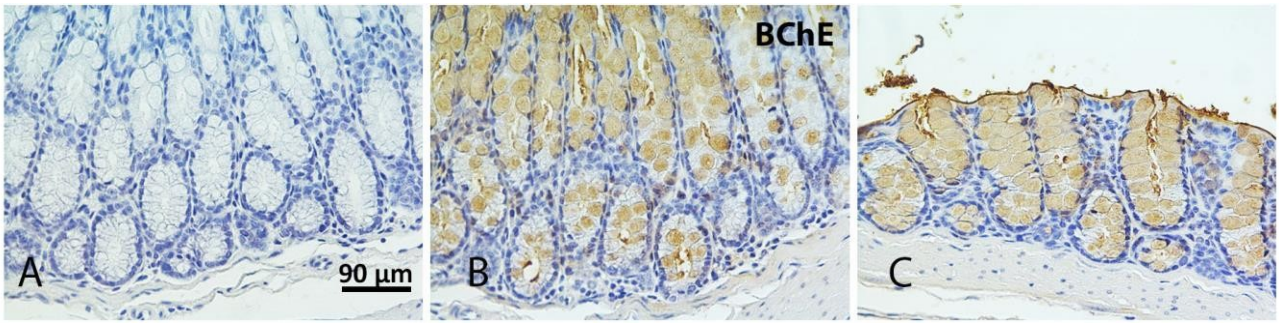

Figure 25| *Distribution of BChE in the mouse colon*. A) Negative control; B, C) Distribution of BChE in mouse colonic mucosa. Scale bar: 90 µm A, B, C.

DISCUSSION

BChE is an hydrolytic enzyme belonging to the carboxylesterases superfamily present in all mammals. In this thesis the distribution of this enzyme all along the mouse gastrointestinal tract was performed. To validate the antibody we confirmed some concepts already known from literature: BChE presence and distribution in the mouse liver and its increase subsequent to *HFD* treatment (Siskova et al., 2016), as well as its distribution in some *nuceli* of the mouse brain (Reid et al., 2013). By immunohistochemistry we described the distribution of BChE in the mouse gastro-intestinal tract: in the liver, in cholangiocytes and hepatocytes, in the keratinized esophageal epithelium as well as in the ductal portion of the salivary glands, in the forestomach and in the glandular stomach, in the exocrine and endocrine pancreas and in the spleen, as well as in the small and the large intestine. This complete gastro-intestinal characterization allow us to speculate on possible BChE physiological functions which have not been clarified yet.

This is the first report of BChE presence in liver cholangiocytes. Liver is innervated by the vagus nerve which through ACh modulates bile formation and modification (Tabibian et al., 2013). The presence of BChE in cholangiocytes could be linked to the regulation of ACh-mediated stimulation of those processes. As a matter of fact, ACh induces $HCO3^-$ secretion through the activation of muscarinic M1 and M3 receptors expressed on the cholangiocytes basolateral plasma membrane (Alvaro et al., 1997). Since cholangoicytes have also been described as part of the hepatic stem cell niche in liver (Kordes and Haussinger, 2013), a role for BChE in the maintenance of liver homeostasis and cellular turn over could be proposed. The secretory and proliferative processes of cholangiocytes are regulated by numerous gastrointestinal hormones and/or neuropeptides including ACh, and it has been demonstrated that ACh, via M3-receptor subtypes and adenyl cyclase modulation, acts as a trophic/growth factor in proliferating cholangiocytes (LeSag et al., 1999) suggesting an involvement of BChE.

The presence of the cholinergic machinery outside the nervous system has been firstly reported in 1978 by Sastry and Sadavongvivad (Sastry and Sadavongvivad, 1978). Our results show the presence of BChE in stem cell niches such as in the already described hepatic cholangiocytes, in the neck portion of the glandular stomach as well as in the intestinal Paneth cells (Barker et al., 2010; Karam, 1993; Sato et al., 2011; Tian et al., 2011). Gastrointestinal organs are constantly in contact with nutrients, metabolites and resident bacteria, which on the one hand are indispensable for health, but on the other cause constant damages to the epithelium, including genetic alterations (Alonso and Yilmaz, 2018), and require a strict controlled homeostasis regulation to avoid oncogenic

transformation. As ACh acts as a trophic molecule towards stem cells proliferation linking to its muscarinic (mostly M3) (Slack, 2000) and nicotinic receptors (Takahashi et al., 2018; Zhao et al., 2014), BChE detection could modulate ACh activity. Indeed, there are numerous evidences proving the proliferative role of ACh on gastrointestinal stem cells. Middelhoff and coworkers, demonstrated the interaction between M3 signaling and Lgr5+ ISC maintenance (Middelhoff et al., 2020), showing how the deletion of M3 reduced the number of Lgr5+ intestinal stem cells. In addition, physiological homeostasis has also been shown to be regulated by nAChR involved in regulation of epithelial cell growth, migration, differentiation, and inflammation processes in various mammalian non-neuronal cells such as epithelial, endothelial and immune cells (Takahashi, 2020; Wessler and Kirkpatrick, 2008). Moreover, it has been demonstrated the existence of α2β4 receptor subtype in Paneth cells, corroborating the hypothesis of nAChRs as regulator of stem cell proliferation and differentiation (Takahashi et al., 2018). It has also been reported that a deficiency of the β4 subunit causes a decrease in crypt size and ISC proliferation and differentiation (Takahashi et al., 2020). ACh generated from tuft cells in the gastric epithelium, regulates gastric epithelial proliferation and regeneration, as well as the clonal expansion of $Lgr5^{+}$stem cells via the muscarinic receptor subtype 3, playing a pivotal role in the stem cell niche (Hayakawa et al., 2017; Zhao et al., 2014). Moreover, also vagal innervation is fundamental in the regeneration process of gastric epithelium. It has been described a markedly reduced tumor incidence and progression after surgical or pharmacological denervation of the stomach, suggesting that vagal innervation may contribute to the gastric tumorigenesis via M3 receptor-mediated Wnt signaling in stem cells (Zhao et al., 2014). All of those evidences highlighted the importance of BChE and its fundamental role in balancing cell renewal in the gastrointestinal tract.

Another important novelty arising from our study is the presence of BChE in the parietal cells of the mouse stomach. Interestingly, it has been described the role of ACh as one of the main stimulators of parietal cells secretion (Schubert and Shamburek, 1990), and mucus secretion from goblet cells of the more distal portion of the intestine, the colon (Specian and Neutra, 1980). Those concepts allow us to hypothesize another physiological function for BChE: the control of gastric secretions and the digestion of ingested ACh present in foods. The importance of the vagus nerve in stimulating acid secretion was for the first time elaborated by Pavlov, who showed that vagotomy decreased basal acid secretion (Engevik et al., 2020)Parietal cells receive direct vagal stimulation, usually mediated by cholinergic neurons of the enteric nervous system. ACh, acting through muscarinic M3

receptors (Aihara et al., 2003), induces the mobilization of Ca^{2+} from cellular stores via phospholipase-C activation and inositol triphosphate elevation (Hou and Schubert, 2006), and leads to the HCl secretion from the apical cell membrane. The acidic gastric juice is fundamental since it acts as a barrier against ingested pathogens making enzymatic digestion possible (Arin et al., 2017), and allows the stomach to fulfils important tasks in the mechanical and chemical digestion of food. Hydrochloric acid secreted from gastric parietal cells generates a strongly acidic environment into the gastric lumen that reaches the pH values of 1-2 (Quigley and Turnberg, 1987) which kills food-derived bacteria, enables food digestion, and promotes absorption of minerals including phosphate, calcium, and iron. Even if great amounts of acid secretions are needed to digestive processes, on the other hand high levels of acid secretion also represent a potentially harmful material to the integrity of the gastric mucosa, which, by its side, must maintain a strict balance between acid secretion and mechanisms of mucosal protection (Engevik et al., 2020). To regulate and balance acid secretions, parietal cells have to integrate an intricate network of paracrine (histamine, somatostatin), endocrine (gastrin, somatostatin), and neural components (ACh and others), thus the communication between specialized cells of the gastric mucosa (ECL cells in the body and fundus, G cells in the antrum and D cells in the antrum, body, and fundus) and neurons (Arin et al., 2017) results fundamental. Moreover, parietal cells not only govern gastric acid secretion, but under ACh stimulation also produce growth factors crucial for cell maturation (Baratta et al., 2019). Numerous studies over the past decade have recognized the involvement of parietal cells in physiological mucosal homeostasis. Loss of parietal cells, or oxyntic atrophy, is the pathological condition mostly associated with gastric cancer (El-Zimaity et al., 2002). Hence, BChE detected in gastric parietal cells could be an important regulator of gastric acid secretion but could also act on the ACh stimulation finally inhibiting the growth factors released by parietal cells.

Apart from ACh, also ghrelin, released from the oxyntic mucosa of the stomach, regulates gastric acid secretion performed by parietal cells (Engevik et al., 2020). Our third hypothesis, suggested by BChE distribution in the glandular portion of the stomach, could be a possible role for this enzyme towards the peptide ghrelin. It has been demonstrated how peripheral (Masuda et al., 2000) and intracerebroventricular (Date et al., 2001) administration of ghrelin stimulates gastric acid secretion through the vagus nerve in rats. Our findings revealed a spatial relationship between ghrelin producing cells and parietal cells containing BChE in gastric glandular epithelium. Moreover, a novel and recently emerged role for BChE towards ghrelin has been clarified (Chen et al., 2016; De Vriese et al., 2004; Sato et al., 2011). After the discovery of the hydrolytic role of BChE towards the hunger

hormone ghrelin (Chen et al., 2016; De Vriese et al., 2004), its relevance has grown significantly, linked to obesity and appetite regulation. As stomach is the source of ghrelin (Boudesteyn et al., 2002) and we have demonstrated a spatial correlation between the two cytotypes, we could suggest that BChE may exerts its hydrolytic function towards ghrelin through a paracrine action, not only in plasma, as has already been shown (Chen et al., 2015), but directly into the gastric mucosa preventing also ghrelin secretion at a plasmatic level. Hence, a dual role of BChE acting on parietal cells in relation to ghrelin could be suggested: a direct effect, through the cut off of ghrelin at a parenchymal level or an indirect effect, towards ghrelin stimulating process of acid secretion made by parietal cells.

Another possible physiological function of BChE could be proposed studying its producing cells and it is linked to its detoxifying action. BChE has been studied for a long time for its role as a detoxifying agent on compounds such as chemical nerve agents developed in twenties, and pesticides, but its detoxifying characteristics have never been related to dietary compounds until quite recently (Johnson and Moore, 2012). Its expression all along the gastrointestinal tract shown herein, as well as the already known presence in the major organs of entry, among which lungs, liver and serum (Jbilo et al., 1994), support the concept that it may operate as a detoxifier. BChE, in fact, perfectly fits the criteria of an enzyme of detoxification proposed by Ziegler (Ziegler, 1991): concentration in the major organs of entry, broad substrate range, and variation in concentration between different species in response to differences in diet (Johnson and Moore, 2012). Its large acyl pocket and active site gorge allow the accommodation of a larger range of substrates in respect to its brother enzyme AChE, including traditional anti-ChEs, such as organophosphates and carbamates, as well as other compounds, such as cocaine (Xie et al., 1999) and heroin (Qiao et al., 2014) or the already described ghrelin. It is also possible to hypothesize that serum AChE may as well act as a detoxifier since it is structurally related to BChE, it is expressed in large quantities in the erythrocyte membrane and it is inhibited by pesticide exposure (Fontoura-da-Silva and Chautard-Freire-Maia, 1996), but, despite of this, AChE specialized structure, limits its substrates to relatively small compounds, specifically, to anti-ChEs that were accurately designed as AChE targets (such as pesticides and nerve toxic agents). To strengthen the concept of BChE, and not AChE, involvement in the detoxifying process, it has been demonstrated that some individuals, subsequently to pesticides exposures, do not show evidence of poisoning, but only reduced BChE activity (Lockridge and Masson, 2000). In addition, it has been shown that treating animals with BChE before exposure to nerve agents could be protective against those organophosphate compounds (Broomfield et al., 1991; Saxena et al., 2006).

On the other side, BChE knockout mouse, lacking the enzyme, have been found to be more sensitive to poisoning by these agents (Duysen et al., 2007). In light of all those evidences, it is clear the relationship between the enzyme and detoxification processes. Interestingly, numerous anti-ChEs exist in the natural environment and many of them derive from plants metabolism and are used in nature from plants themselves as a protection tool against herbivory. However, also alkaloids, such as those found in the Solanaceae family, as well as various carbamates, such as physostigmine from the calabar bean, belongs to anti-ChEs category (Bodur and Cokugras, 2005; Zhang and Casida, 2002). Moreover, exposure to dietary anti-ChEs is known to influence the activity of BChE in serum. As a matter of fact, it has been observed that humans eating a meal of potatoes (which contain the glycoalkaloids α-solanine, α-chaconine and solanidine) displayed reduced serum BChE activity (McGehee et al., 2000). Measurement of BChE activity has also been used for the detection of toxins, specifically the solanaceous alkaloids (Arkhypova et al., 2003). The presence of an ubiquitous bioscavenger such as BChE in the intestine, stomach and serum, organs whose main tasks are ascribed to the transport and absorption of compounds through their epithelia, could be fundamental as a first barrier against natural ingested poisons. Furthermore, botanical anti-ChEs are not only a source for the development of pesticides (i.e., carbamate physostigmine from the calabar bean), but are also employed for therapeutics (i.e., galanthamine, a specific AChE inhibitor, used for the treatment of Alzheimer's disease) (Orhan, 2012). Since chronic exposure to anti-ChEs has been linked to various adverse effects, from teratogenicity to the development of a variety of human cancers (Alavanja and Bonner, 2012), it is possible to understand the critical importance of the presence of detoxicant mechanisms carried out by physiological bioscavengers, such as BChE.

In conclusion, BChE initially considered to lack a physiological function apart from serving as a "backup" enzyme for AChE for its hydrolytic action towards ACh, turns out to be an interesting enzyme. Even though the significance of the distribution of BChE in gastrointestinal cells remains to be elucidated, this thesis work could provide a detailed background for further experimental projects and studies aiming to clarify the role of this enzyme in the mouse GI-tract.

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