学位論文（博士）

Immunohistochemical phenotypes of huntingtin-associated protein 1 in the enteric nervous system of adult mouse

(成獣マウスの腸管神経系における HAP1 の免疫組織化学的発現形態)

氏名　TARIF ABU MD MAMUN

所属　山口大学大学院医学系研究科

医学専攻 (神経解剖学講座)

令和 4 年 5 月

Table of Contents

Chapter I

General Abstract

Huntingtin-associated protein 1 (HAP1) is a neural huntingtin interactor and being considered as a core molecule of stigmoid body (STB). Brain or spinal cord regions with abundant STB/HAP1 expression are usually spared from neurodegeneration, whereas the regions with little STB/HAP1 expression are always neurodegenerative targets. The enteric nervous system (ENS) can act as a potential portal for pathogenesis of neurodegenerative disorders. To date, the expression of HAP1 and its neurochemical characterization have never been examined there. In the current study, we determined the expression and immunohistochemical phenotypes of HAP1 in ENS of adult rodents using Western blotting and light/fluorescence microscopy. HAP1 immunoreactivity was strongly expressed in both myenteric and submucosal plexuses of ENS. STBs were observed in the cytoplasm of most of the HAP1-immunoractive (ir) cells in ENS. In myenteric plexus, a large number of calretinin, calbindin, NOS, VIP, ChAT, SP, somatostatin, and TH-ir neurons showed HAP1 immunoreactivity. In contrast, most of the CGRP-ir neurons were devoid of HAP1-immunoreactivity. In submucosal plexus, almost all the cholinergic secretomotor neurons containing ChAT/ CGRP/ somatostatin/ calretinin, non-cholinergic secretomotor neurons containing VIP/TH/calretinin and vasodilator neurons containing VIP/calretinin express HAP1. Our current study is the first to clarify that HAP1 is highly expressed in excitatory motor neurons, inhibitory motor neurons, and interneurons but almost absent in sensory neurons in myenteric plexus. While, HAP1 is expressed in all neuronal subgroups of Meissner's plexuses. These suggest that due to lack of putative STB/HAP1 protectivity, the sensory neurons (Dogiel type II) might be more vulnerable to neurodegeneration than STB/HAP1-expressing Dogiel type I neurons in myenteric plexus and

secretomotor/vasodilator neurons in Meissner's plexuses. Our current results may reflect the involvement of HAP1 in modulation of excitatory and inhibitory motor neuron functions in myenteric plexus and the secretomotor and vasodilator functions of submucosal neurons. It will be of great interest to elucidate the physiological or pathological roles of HAP1 in ENS. Our current results might lay a basic foundation for future studies that seek to clarify the physiological/pathological effects of STB/HAP1 in the ENS.

Chapter II

Background of research

2.1 HAP1 is a determinant marker for the stigmoid body (STB)

Huntingtin-associated protein 1 (HAP1) is a core component of the stigmoid body (STB) and known as a neuroprotective interactor with causal agents for various neurodegenerative diseases. It was initially identified as a polyglutamine (polyQ) length-dependent interactor of huntingtin (htt), the causal agent responsible for Huntington's disease (Li et al. 1995). Transfection of HAP1 cDNA into different cultured cells can also induce the development of STB (Li et al. 1998a; Takeshita et al. 2006; Fujinaga et al. 2007, 2011), and HAP1 is thus considered as a determinant marker for STB (Li et al. 1998a; Fujinaga et al. 2007; Islam et al. 2017; Wroblewski et al. 2018). STB was first reported in our previous studies as a spherical to ovoid shaped (0.5–3 μm in diameter), non-membranous, granular to fuzzy textured cytoplasmic inclusion (Shinoda et al. 1992, 1993).

2.2 HAP1 protection hypothesis in central nervous system

In normal rodents, STB/HAP1 is highly distributed in the preoptic area, amygdala, hypothalamus, spinal dorsal horn and intermediolateral horn or in dorsal root ganglion (Kamei et al. 2001; Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Islam et al. 2012, 2017, 2020; Wroblewski et al. 2018; Chen et al. 2020). Intriguingly, these regions are usually spared from neurodegeneration in neurodegenerative diseases. However, the target regions in different neurodegenerative disease such as cortex, thalamus, striatum, cerebellum, and spinal motoneurons have little or no HAP1-expression (Fujinaga et al.

2004; Islam et al. 2012, 2017, 2020a; Wroblewski et al. 2018). The mounting evidence suggests that STB/HAP1 has protective functions against cell death induced by htt (Koga et al. 2002; Li et al. 2003; Metzger and Rong 2008; Liu et al. 2020). Furthermore, STB/HAP1 can also bind to androgen receptor (AR) in a polyQ-length dependent manner, sequestering polyQ-expanded AR derived from spinal and bulbar muscular atrophy (SBMA) more strongly and suppressing SBMA-mutant AR-induced apoptosis via inhibition of its nuclear translocation from the cytoplasm (Takeshita et al. 2006). In addition, STB/HAP1 can also interact with other polyQ-expanded gene products such as ataxin-3 in spinocerebellar ataxia (SCA) type 3 (Takeshita et al. 2011) and TATA-binding protein in SCA type 17 (Prigge and Schmidt, 2007), suppressing their nuclear translocation. Furthermore, apoptosis or neurodegeneration is facilitated in the hypothalamus of Hap1-KO mice (Li et al. 2003). This line of data strongly supports the ‘‘STB/HAP1 protection hypothesis” (Fujinaga et al. 2004) in which HAP1 is thought to raise the threshold of vulnerability for neurodegeneration, render increased stability to neurons, and consequently protect against apoptosis and cell death in several neurodegenerative diseases (Kamei et al. 2001; Koga et al. 2002; Li et al. 2003; Fujinaga et al. 2004; Metzger et al. 2008; Takeshita et al. 2006, 2011; Islam et al. 2017). Regarding physiological functions, HAP1 can act as a mediator of feeding behaviors, as a sensor for insulin signals in feeding control or as a stabilizer of glucocorticoid receptor in stress response (Chan et al. 2002; Dragatsis and Zeitlin 2004; Sheng et al. 2006; Lin et al. 2010; Niu et al. 2011; Chen et al. 2020).

2.3 HAP1 protection hypothesis in peripheral nervous system

In addition to the brain and spinal cord in central nervous system (CNS) (Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Islam et al. 2012, 2017; Wroblewski et al. 2018; Chen et al. 2020), STB/HAP1 is also expressed in the peripheral system including

dorsal root ganglion (Islam et al. 2020a). HAP1 and markers for nociceptive or mechanoreceptive neurons showed that about 70-80 % of CGRP-, SP-, CB-, NOS-, TRPV1-, CR- and PV-ir neurons expressed HAP1. In contrast, HAP1 was completely lacking in TH-ir neurons in DRG (Islam et al. 2020a). It clarified that HAP1 is highly expressed in nociceptive/proprioceptive neurons but absent in light-touch-sensitive TH neurons, suggesting the potential importance of HAP1 in pain transduction and proprioception. Our result suggest that the "HAP1 protection hypothesis," which has previously been proposed for the brain and spinal cord in the central nervous system (Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Islam et al. 2012, 2017), might also be applied to the DRG in the peripheral nervous system. HAP1 expression might increase the threshold of vulnerability to neurodegeneration and confer beneficial stability to different sensory neurons excluding the touch-sensitive mechanoreceptive ones in the DRG. Furthermore, STB/HAP1 is also expressed in enteroendocrine glands in the mucosa of the gastrointestinal tract (Liao et al. 2005; Li et al. 2019; Yanai et al. 2020). In addition to the CNS, enteric nervous system (ENS) is also an important target of certain neurodegenerative disorders (Chalazonitis and Rao 2018). It might be possible that HAP1 is also present in the ENS and might increase the threshold of vulnerability to enteric neuropathology.

2.4 Enteric nervous system

The enteric nervous system (ENS) is embedded in the wall of the gastrointestinal tract and contains polarized neural circuits responsible for controlling a wide variety of gastrointestinal functions independently from the CNS (Furness 2012; Furness et al. 2014). ENS is often called as the "second brain" in the gastrointestinal tract (Gershon et al. 1999). It shares many features with the brain. ENS is the most complex and largest unit of the peripheral nervous system. It has been treated as the third division of the

autonomic nervous system in addition to the sympathetic and parasympathetic divisions (Furness and Costa 1980; Furness 2000). Intriguingly, in our recent study, we have reported that almost all the spinal preganglionic sympathetic and parasympathetic neurons express HAP1(Islam et al. 2017). In addition to the CNS, ENS is also an important target of certain neurodegenerative disorders (Chalazonitis and Rao 2018). ENS is also regarded as a potential portal for the pathogenies of neurodegenerative diseases (Kuwahara et al. 2020; Sharon et al. 2016). It is possible that HAP1 might be involved in modulating or protecting certain functions of ENS. In this context, it becomes important to examine the distribution of HAP1 in ENS and to clarify its neurochemical phenotypes.

2.5 The Classification of enteric neurons in myenteric plexus

The ENS contains many different types of neurons which can be classed according to their morphology, electrical properties, function, or neurochemistry.

2.5.1 Morphological classification of enteric neurons

Shape, which is one of the identifying features of neurons, both in the central nervous system and in peripheral ganglia (Cajal 1911, Brehmer 2006). On the basis of the shape of cell body and the number of long processes originating from it, the enteric neurons can also be categorized into Dogiel types I, II, and III. Dogiel type I neurons contain one long process and Dogiel type II neurons have several long processes originating from their cell body. Dogiel type I groups include mainly motor neurons and interneurons, whereas, Dogiel type II groups include sensory neurons (Furness et al. 1998). Unlike the first two types, Dogiel's description of type III neurons has not been definitively confirmed by current methodologies, nonetheless type III is occasionally used to define filamentous neurons with long branched processes (Furness, 2006).

2.5.2 Neurochemical coding of myenteric neurons

The enteric motor neurons can further be categorized as excitatory and inhibitory ones. The excitatory motor neurons cause contraction by releasing excitatory neurotransmitters (Choline acetyltransferase, ChAT; Substance P, SP) and inhibitory motor neurons cause relaxation by releasing inhibitory neurotransmitters (Nitric oxide synthase, NOS; Vasoactive intestinal peptide, VIP) to the smooth muscle (Brookes 2001; Qu et al. 2008). Besides these neuronal subpopulations, prominent roles in ENS have been attributed to the calcium binding protein (calbindin, calretinin), calcitonin gene-related peptide (CGRP), tyrosine hydroxylase (TH) and somatostatin (Qu et al. 2008; Mitsui 2009; Masliukov et al. 2017). In mouse, ChAT is present in about 55-63%, NOS is expressed in about 29%, calretinin is present in approximately 52%, calbindin is expressed in around 28%, substance P is present in about 29%, CGRP is expressed about 28%, and TH is present less than 0.5% of total enteric neurons (Vanden Berghe et al. 1999; Sang 1998; Qu et al. 2008; Van Op Den Bosch et al. 2008; Furness 2012).

2.5.3 Functional classes of myenteric neurons

The ENS is comprised mainly of an outer plexus (Auerbach's or myenteric) between the intestinal muscular layers and an inner plexus (Meissner's or submucosal) in the submucosal layer. Neurons of myenteric plexus mainly involve in controlling smooth muscle motility, whereas the neurons of the Meissner's plexus primarily involve in regulation of secretion (Furness 2006). Enteric neurons are functionally distinct and they differ in terms of morphology, electrophysiological properties, and neurochemical coding (Furness 2000; Mazzoni et al. 2020). Myenteric neurons can be classified as motor neurons (excitatory and inhibitory), sensory neurons (intrinsic primary afferent neurons, IPANs), and interneurons (Furness et al. 1998; Smolilo et al. 2019). On the

other hand, Neurons of Meissner's plexus have been classified as cholinergic secretomotor, non-cholinergic secretomotor, and vasodilator groups (Mongardi Fantaguzzi et al. 2009). Cholinergic secretomotor neurons express ChAT, CGRP, somatostatin, and calretinin. Non-cholinergic secretomotor neurons express VIP, NOS, TH, and calretinin. Vasodilator neurons express VIP, and calretinin. Calretinin is expressed in all three subtypes of submucosal neurons, whereas, VIP is expressed in both non-cholinergic secretomotor and vasodilator neurons (Lomax and Furness, 2000; Harrington et al. 2005; Mongardi Fantaguzzi et al. 2009; Girotti et al. 2013).

2.6 The Classification of enteric neurons in submucosal plexus

Submucosal ganglia have mainly three types of neurons, VIP-immunoreactive non-cholinergic secretomotor neurons (~30% of neurons), VIP-immunoreactive non-cholinergic vasomotor neurons (~20% of neurons), and cholinergic secretomotor neurons (~45% of neurons) (Mongardi Fantaguzzi et al. 2009). Based on neurochemical phenotypes, submucosal ganglia contain cell bodies of VIP/ NPY/ TH/ calretinin non-cholinergic secretomotor neurons, VIP/ NPY/ calretinin vasodilator neurons, ChAT/ CGRP/ somatostatin/ calretinin cholinergic secretomotor neurons and small populations of cholinergic and non-cholinergic neurons whose targets have yet to be identified. No evidence for the presence of type-II putative intrinsic primary afferent neurons was found in the submucosal plexus of mouse intestine (Mongardi Fantaguzzi et al. 2009).

Chapter III

Research objectives

The expression of HAP1-immunoreactivity and its neuroanatomical distribution have never been examined in the ENS. Neurochemical phenotypes of HAP1-ir structures in the ENS in reference to motor neurons (excitatory and inhibitory), sensory neurons (intrinsic primary afferent neurons, IPANs), and interneurons in myenteric plexus and secretomotor neuron and vasodilator neurons in submucosal plexus have also not been elucidated yet. In the current study, we set out to examine the expression of HAP1 throughout different segments of the ENS of adult mouse. We also aimed to clarify the neurochemical phenotypes of HAP1-ir cells in relation to the neurochemical markers of enteric neurons.

This thesis has two major aims.

I. Immunohistochemical expression and neurochemical phenotypes of huntingtin-associated protein 1 in the myenteric plexus of mouse gastrointestinal tract.
II. Neurochemical phenotypes of huntingtin-associated protein 1 in reference to secretomotor and vasodilator neurons in the Meissner's plexuses of rodent small intestine.

Chapter IV

Detailed of research objectives I

Immunohistochemical expression and neurochemical phenotypes of huntingtin-associated protein 1 in the myenteric plexus of mouse gastrointestinal tract.

4.1 Abstract

Huntingtin-associated protein 1 (HAP1) is a neural huntingtin interactor and being considered as a core molecule of stigmoid body (STB). Brain/spinal cord regions with abundant STB/HAP1 expression are usually spared from neurodegeneration in stress/disease conditions, whereas the regions with little STB/HAP1 expression are always neurodegenerative targets. The enteric nervous system (ENS) can act as a potential portal for pathogenesis of neurodegenerative disorders. However, ENS is also a neurodegenerative target in these disorders. To date, the expression of HAP1 and its neurochemical characteriza- tion have never been examined there. In the current study, we determined the expression of HAP1 in the ENS of adult mice and characterized the morphological relationships of HAP1-immunoreactive (ir) cells with the markers of motor neurons, sensory neurons, and interneurons in the myenteric plexus using Western blotting and light/fluorescence microscopy. HAP1- immunoreaction was present in both myenteric and submucosal plexuses of ENS. Most of the HAP1-ir neurons exhibited STB in their cytoplasm. In myenteric plexus, a large number of calretinin, calbindin, NOS, VIP, ChAT, SP, somatostatin, and TH-ir neurons showed HAP1-immunoreactivity. In contrast, most of the CGRP-ir neurons were devoid of HAP1-immunoreactivity. Our current study is the first to clarify that HAP1 is highly expressed in excitatory motor neurons, inhibitory motor neurons, and interneurons but

almost absent in sensory neurons in myenteric plexus. These suggest that STB/HAP1-ir neurons are mostly Dogiel type I neurons. Due to lack of putative STB/HAP1 protectivity, the sensory neurons (Dogiel type II) might be more vulnerable to neurodegeneration than STB/HAP1-expressing motoneurons/interneurons (Dogiel type I) in myenteric plexus.

4.2 Introduction

Huntingtin-associated protein 1 (HAP1) is a neurocytoplasmic protein. It was initially identified as a polyglutamine (polyQ) length-dependent interactor of huntingtin (htt), the causal agent responsible for Huntington's disease (Li et al. 1995). HAP1 is also regarded as a determinant marker for the stigmoid body (STB) (Li et al. 1998; Gutekunst et al. 1998; Fujinaga et al. 2007; Takeshita et al. 2011; Yanai et al. 2020). STB was first reported in our previous studies as a spherical to ovoid shaped (0.5–3 μm in diameter), non-membranous, granular to fuzzy textured cytoplasmic inclusion (Shinoda et al. 1992, 1993). The mounting evidence suggests that STB/ HAP1 has protective functions against cell death induced by htt (Koga et al. 2002; Li et al. 2003; Metzger and Rong 2008; Liu et al. 2020). Furthermore, STB/HAP1 can also protect apoptosis/cell death induced by the causal agents of some other polyQ diseases, including spinal and bulbar muscular atrophy (Takeshita et al. 2006), Machado-Joseph disease (Takeshita et al. 2011), Joubert syndrome (Sheng et al. 2008), and spinocerebellar ataxia type 17 (Prigge and Schmidt 2007).

In normal rodents, STB/HAP1 is highly distributed in the preoptic area, retrosplenial-retrohippocampal area, hypo- thalamus, amygdala, spinal dorsal horn, and intermediolateral horn or in dorsal root ganglion (Kamei et al. 2001; Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Islam et al. 2012, 2017, 2020a; Wroblewski et al.

2018; Chen et al. 2020). Intriguingly, these regions are usually spared from neurodegeneration in neurodegenerative diseases. However, the target regions in different neurodegenerative diseases such as cortex, thalamus, striatum, cerebellum, and spinal motoneurons have little or no HAP1 expression (Fujinaga et al. 2004; Islam et al. 2012, 2017, 2020a; Wroblewski et al. 2018). These indicate that STB/HAP1 can raise the threshold of vulnerability to neurodegeneration. STB/HAP1 may give out increased stability to neurons and protect against apoptosis/cell death in certain neurodegenerative disorders. This phenomenon has been coined as STB/HAP1 protection hypothesis (Kamei et al. 2001; Fujinaga et al. 2004; Takeshita et al. 2006; Metzger and Rong 2008; Islam et al. 2017). Regarding physiological functions, HAP1 can act as a mediator of feeding behaviors, as a sensor for insulin signals in feeding control, or as a stabilizer of glucocorticoid receptor in stress response (Chan et al. 2002; Dragatsis and Zeitlin 2004; Sheng et al. 2006; Lin et al. 2010; Niu et al. 2011; Chen et al. 2020).

In addition to the brain and spinal cord in central nervous system (CNS) (Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Islam et al. 2012, 2017; Wroblewski et al. 2018; Chen et al. 2020) or dorsal root ganglion in peripheral nervous system (Islam et al. 2020a), STB/HAP1 is also expressed in the peripheral organs including thyroid gland, pituitary gland, and pancreatic islets (Dragatsis et al. 2000; Liao et al. 2005). Furthermore, STB/HAP1 is also expressed in enteroendocrine glands in the mucosa of gastrointestinal tract (Liao et al. 2005; Li et al. 2019; Yanai et al. 2020). The enteric nervous system (ENS) is embedded in the wall of the gastrointestinal tract and contains polarized neural circuits responsible for controlling a wide variety of gastrointestinal functions independently from the CNS (Furness 2012; Furness et al. 2014). ENS is often called as the “second brain” in the gastrointestinal tract (Gershon

et al. 1998). It shares many features with the brain. ENS is the most com- plex and largest unit of the peripheral nervous system. It has been treated as the third division of the autonomic nervous system in addition to the sympathetic and parasympathetic divisions (Furness and Costa 1980; Furness 2000). Intriguingly, in our recent study, we have reported that almost all the spinal preganglionic sympathetic and parasympathetic neurons express HAP1 (Islam et al. 2017). In addition to the CNS, ENS is also an important target of certain neurodegenerative disorders (Chalazonitis and Rao 2018). ENS is also regarded as a potential portal for the pathogenies of neurodegenerative diseases (Kuwahara et al. 2020; Sharon et al. 2016). It is possible that HAP1 might be involved in modulating or protecting certain functions of ENS. In this context, it becomes important to examine the distribution of HAP1 in ENS and to clarify its neurochemical phenotypes.

The ENS is comprised mainly of an outer plexus (Auerbach's or myenteric) between the intestinal muscular layers and an inner plexus (Meissner's or submucosal) in the submucosal layer. Neurons of myenteric plexus are mainly involved in controlling smooth muscle motility, whereas the neurons of the Meissner's plexus are primarily involved in regulation of secretion (Furness 2006). Enteric neurons are functionally distinct and they differ in terms of morphology, electrophysiological properties, and neurochemical coding (Furness 2000; Mazzoni et al. 2020). Enteric neurons can be classified as motor neurons (excitatory and inhibitory), sensory neurons (intrinsic primary afferent neurons, IPANs), and interneurons (Furness et al. 1998; Smolilo et al. 2019). On the basis of the shape of cell body and the number of long processes originating from it, the enteric neurons can also be categorized into Dogiel type I and type II groups. Dogiel type I neurons contain one long process and Dogiel type II neurons have several long processes originating from their cell body. Dogiel

type I groups include mainly motor neurons and interneurons, whereas Dogiel type II groups include sensory neurons (Furness et al. 1998).

The enteric motor neurons can further be categorized as excitatory and inhibitory ones. The excitatory motor neurons cause contraction by releasing excitatory neurotransmitters (choline acetyltransferase, ChAT; substance P, SP) and inhibitory motor neurons cause relaxation by releasing inhibitory neurotransmitters (nitric oxide synthase, NOS; vasoactive intestinal peptide, VIP) to the smooth muscle (Brookes 2001; Qu et al. 2008). Besides these neuronal subpopulations, prominent roles in ENS have been attributed to the calcium-binding protein (calbindin, calretinin), calcitonin gene-related peptide (CGRP), tyrosine hydroxylase (TH), and somatostatin (Qu et al. 2008; Mitsui 2009; Masliukov et al. 2017). In mouse, ChAT is present in about 55–63%, NOS is expressed in about 29%, calretinin is present in approximately 52%, calbindin is expressed in around 28%, substance P is present in about 29%, CGRP is expressed about 28%, and TH is present less than 0.5% of total enteric neurons (Vanden Berghe et al. 1999; Sang 1998; Qu et al. 2008; Van Op Den Bosch et al. 2008; Furness 2012).

To date, however, the expression of HAP1-immunoreactivity and its neuroanatomical distribution have never been examined in the ENS. Neurochemical phenotypes of HAP1-immunoreactive (ir) structures in the ENS in reference to above mentioned neuronal markers have also not been elucidated yet. In the current study, we set out to examine the expression of HAP1 throughout different segments of the ENS of adult mouse. We also aimed to clarify the neurochemical phenotypes of HAP1-ir cells in relation to the neurochemical markers of motor neurons (both excitatory and

inhibitory), interneurons, and IPANs in the myenteric plexus of ENS (stomach, duodenum, and colon).

4.3 Materials and methods

4.3.1 Animals and ethical statements

C57BL/6J adult mice (8-10 weeks of age) of either sex, were collected from Japan SLC Inc. (Shizuoka, Japan). They were maintained in a cage in environmentally controlled room with a 12-h light/dark cycle (lights on 08:00-20:00) and provided water and a standard pellet diet *ad libitum*. Necessary cares were taken to keep down the discomfort of animals and the number of mice used.

4.3.2 Tissue section preparation

The mice were perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4) under anesthesia with sodium pentobarbital (60-80mg/kg). The entire gastrointestinal tract was taken off and post-fixed for overnight at 4°C in same fixative used for perfussion. For free floating sections different segment of gastrointestinal tract were transferred into 30% sucrose solution for one week at 4°C for cryoprotection. The experimental segments (stomach-corpus, duodenum, mid colon) were cut into 1-1.5cm full thickness rings, embedded in optimal cutting temperature medium (Sakura Finetek USA, Inc., Torrance, CA, USA) and then blast-freeze was done using powdered dry ice. Finally, the tissues were sectioned frontally at a thickness of 30μm.

4.3.3 Whole mount preparation

After post fixation described above, segments (1-2 cm in length) from esophagus, stomach (corpus), duodenum, jejunum, ileum, cecum, proximal colon, distal colon, and

rectum were cut and pinned flat in the Sylgard-lined Petri dish. The fixative was then cleared off with dimethyl sulfoxide saline and phosphate buffer saline (PBS) washes. Using fine dissecting forceps, the mucosa, submucosa, and circular muscle were removed from fixed tissues and whole mounts consisting of the myenteric plexus adhering to the longitudinal muscle were prepared from each segment (Swaminathan et al. 2019).

4.3.4 Primary antibodies

Primary antibodies for Western blotting or immunohistochemistry used in the present research are listed in Table 1. All the antibodies are available commercially. Characterization of the primary antibodies was performed in previous studies or in the current study.

4.3.5 Immunoperoxidase single labeling immunohistochemistry

Free-floating sections or whole mount preparations were processed for immunoperoxidase immunohistochemistry following the protocol described in our previous studies (Islam et al. 2012, 2017; Wroblewski et al. 2018). Sections or whole mount preparations were incubated in PBS containing 10% normal donkey serum (NDS) and 0.3% Triton X-100 for 1h at 4°C. All sections or whole mount preparations then were bleached in a mixture of 1.5% hydrogen peroxide and 50% methanol for 30 min at 4°C. After rinsing in PBS containing 0.3% Triton X-100 and 0.05% NDS (PBST-NDS), sections or whole mount preparations were placed in anti-HAP1 primary antibody (R19, 1:20,000, diluted in 1% PBST-NDS) for 4 days at 20°C. For the preadsorption test, samples were kept in a mixture of primary antibody (HAP1) and blocking peptide against anti-HAP1 antibody in a separate incubation. Samples were

then rinsed in 0.05% PBST-NDS followed by an incubation in biotinylated donkey antigoat IgG (AP180B, Millipore; 1:1,000, diluted in 1% NDS-PBS) for overnight at 4°C. Next, samples were rinsed in 0.05% NDS-PBS and then incubated in peroxidase-conjugated streptavidin (Dako, Glostrup, Den- mark; 1:1,000 diluted in PBS) for 2h at 20°C. After washing with 0.05 M Tris–HCl buffer (pH 7.6), the bound peroxidase was visualized in the sections or in whole mount preparations by reaction for 10-15 min in 3,3′-diaminobenzidine (DAB, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and nickel ammonium sulfate (Sigma– Aldrich, Tokyo, Japan) solution containing 0.0008% hydrogen peroxide. Finally, sections or whole mount preparations were rinsed, mounted on glass slides coated with 0.6% gelatin, and coverslipped with Entellan New (Merck KGaA, Darmstadt, Germany).

4.3.6 Dual-labeling immunofluorescence immunohistochemistry

Whole mount preparations were processed for immunofluorescence immunohistochemistry following the protocol described in our earlier studies (Jahan et al. 2015; Islam et al. 2017, 2021). First, whole mount preparations were washed in PBS and incubated in a blocking solution of PBS containing 10% normal donkey serum (NDS) and 0.3% Triton X-100 for 2h at 4°C. After rinsing in PBST-NDS whole mount preparations were incubated with goat anti-HAP1 antibody (R19, 1:20,000) in combination with one of the following antibodies: rabbit anti-GFAP (1:1,000), rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1; 1: 1,000), rabbit anti-C-Kit (1:1,000), rabbit anti-neuronal nuclei (NeuN; 1: 1,000), rabbit anti-HuD+HuC (1:5,000), rabbit anti-calretinin (1:2,000), rabbit anti-calbindin (1:25,000), rabbit anti-NOS (1:2,000), rabbit anti-VIP (1:5,000), rabbit anti-ChAT (1:2,000), rabbit anti-SP (1:1,000), rabbit anti-somatostatin (1:1,000), rabbit anti-TH (1:1,000), rabbit anti-

CGRP (1:1,000) antibody at 20 ◦C for 4 days (Table 1 for detail about primary antibodies). Colchicine was injected intraperitonially after mixing with 0.9% NaCl saline solution to strengthen the imunoreactivity of neuronal cell bodies in case of using VIP, SP, TH, and CGRP antibodies (Mitsui 2009). Samples were then rinsed in 0.05% PBST-NDS followed by an incubation with a mixture of Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A32790, AB_2762833, Invi-trogen, Rockford, IL, USA; 1:1,000) and Alexa Fluor 594-conjugated donkey anti-goat IgG (A11058, AB_2534105, Invitrogen, Eugene, OR, USA; 1:1,000) secondary antibodies for 2-3hr at 20°C. Finally, the whole mount preparations were mounted on glass slides coated with 0.6% gelatin, and coverslipped with Fluoromount/Plus (K048, Diagonostic Biosystems, Pleasanton, CA, USA).

4.3.7 Western Blotting

Western blotting was also conducted according to the protocol described in our previous studies (Jahan et al. 2015; Islam et al. 2020b). Stomach, duodenum or colon was homogenized using mortar and pestle in liquid nitrogen. Protein concentration for each sample was quantified as previously described (Islam et al. 2017; 2020a). Equal amounts of lysate were separated using 7.5% SDS–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane. The membranes were then blocked in 5% skim milk for 1h at 20 °C. Membranes were incubated with goat polyclonal anti-HAP1 (R19, 1:20,000) or mouse monoclonal anti-□ tubulin (1:20,000) primary antibodies (Table 1) for overnight at 4 °C. Bound antibodies were detected with horseradish peroxidase-linked anti-goat (1:5,000; SC-3851, Santa Cruz Biotechnology) or anti-mouse IgG (1:20,000; GE Healthcare, Buckinghamshire, UK) and visualised by enhanced chemiluminescent detection (ECL select; GE Healthcare). Finally, the

immunoreactive signals were captured using an Amersham Imager 600 (GE Healthcare).

4.3.8 Image processing

Immunoperoxidase staining photomicrographs were captured with an Eclipse E80i photomicroscope (Nikon, Tokyo, Japan) equipped with a color digital camera (USB 2.0, Lumenera Corporation, Ottawa, Canada). Immunofluorescence photomicrographs were taken with a laser-scanning microscope (LSM510; Carl Zeiss, Jena, Germany) with single optical sections (1024x1024 pixels). Photoshop software (Adobe Photoshop Elements 2020, Adobe Systems, Inc. San Jose, CA, USA) was utilized to organize the photographs.

4.3.9 Tissue analyses and cell counting

Analyses of co-expression ratios for HAP1/ neuronal markers or neuronal markers / HAP1 were performed in whole mount myenteric plexus preparations of stomach (corpus), duodenum and mid colon. Only the cells with a clearly visible nucleus were counted. Images were randomly taken and cells were counted in a total of 10 fields ($0.25mm^2$/field) in each mouse. At least 100 neurons from each field were counted (Qu et al. 2008). A total of 4 mice were used for each quantification. Data were expressed as mean ± standard error of the mean (SEM; n =4).

4.4 Results

Immunohistochemistry and Western blotting were performed to examine the expression of HAP1 in the ENS. First, we performed immunohistochemistry of HAP1 using free-floating sections of stomach, duodenum, and colon (Fig. 1). HAP1 was abundantly

expressed in the myenteric plexus of gastrointestinal tract (Fig. 1b, e, h). HAP1-immunureactivity was also found in the Meissner's plexus (Fig. 1 b, e, h). The location of myenteric and Meissner's plexus was clarified by H&E staining in adjacent sections (Fig. 1a,d, g). HAP1-immunoreactivity was abolished in the preadsorption test (Fig. 1c, f, i). Then HAP1-expression in the gastrointestinal tract was confirmed by Western blotting. In Western blotting, both isoforms of HAP1 (approximately 85 kDa for HAP1B and 75 kDa for HAP1A) were identified in the ENS of stomach, duodenum, and colon (Fig. 1j). The expression of HAP1 in the ENS was less than that of hypothalamus. Using the blocking peptide of primary antibody resulted in complete elimination of all the HAP1-ir bands.

In the present study, we particularly focused on the characterization of HAP1-immunoreactivity in the myenteric plexus. For this, the whole-mount preparation was used to confirm the expression pattern of HAP1-ir cells in the myenteric plexus throughout the gastrointestinal tract (Fig. 2). We found that HAP1-ir cells were luxuriantly present in Auerbach's plexus as a mesh-like network in esophagus (Fig. 2a), stomach (Fig. 2b), duodenum (Fig. 2c), jejunum (Fig. 2d), ileum (Fig. 2e), cecum (Fig. 2f), proximal colon (Fig. 2g), distal colon (Fig. 2h), and rectum (Fig. 2i). HAP1-immuoreactivity was mainly present in the cytoplasm of the cells where they often localized to dot like STB. There were also some HAP1-ir cells without or no clear STB.

Next, double-label immunofluorescence staining was performed for HAP1 and GFAP (a marker for enteric glial processes), Iba-1 (a marker for muscularis macrophage), c-kit (a marker for interstitial cells of Cajal) in the duodenum (Fig. 3) or with NeuN (neuronal marker) in stomach, duodenum and colon (Fig. 4). Our present

double staining results showed that almost all the HAP1-ir cells exhibited clear NeuN-immunoreactivity (Fig. 4a-i), while being negative for GFAP (Fig. 3a-c), Iba-1 (Fig. 3d-f) or C-kit (Fig. 3g-i). These results indicated that the HAP1-ir cells demonstrated attributes of neurons but not of enteric glial or Cajal cells. Next, to quantify the number of HAP1-ir neurons in the myenteric plexus, double-label staining of HAP1 was carried out with enteric pan neuronal marker, Hu. The results clearly revealed that 100% HAP1-ir cells were immuno-positive for Hu in stomach, duodenum and colon (Fig. 5), whereas 96.03%, 73.5%, 77.53% of Hu-ir cells were co-expressed with HAP1 in stomach, duodenum, and colon respectively (Fig. 5). Then, to reveal the neurochemical phenotypes of HAP1-ir neurons, we performed double-label immunohistochemistry of HAP1 with the markers of calcium binding proteins, nitrergic/cholinergic motor neurons, interneurons, sympathetic nonadrenergic neurons and sensory neurons, as described below.

4.4.1 Immunohistochemical relationships of STB/HAP1 with calcium binding protein

Among the calcium binding proteins, calretinin and calbindin are most important markers in enteric nervous system (Masliukov et al. 2017). Our current cell counting demonstrated that the co-expression ratio of HAP1 in claretinin-ir neurons were 100% in stomach, 68 - 70% in duodenum and colon and that of calretinin in HAP1-ir neurons were 27% in stomach and 44 - 48% in duodenum and colon (Fig. 6a-i, Table 2). Whereas, our current cell counting demonstrated that the co-expression ratio of HAP1 in calbindin-ir neurons were 100% in stomach, 42 - 46% in duodenum and colon and that of calbindin in HAP1-ir neurons were 24% in stomach and 11 - 13% in duodenum and colon (Fig. 7a-i, Table 2).

4.4.2 Immunohistochemical relationships of STB/HAP1 with markers of excitatory and inhibitory neurons

Excitatory motor neurons of myenteric plexus express ChAT and SP, whereas inhibitory neurons express NOS and VIP (Furness 2000). Our cell counting data indicated that the co-expression ratio of HAP1 in NOS-ir neurons were 96 - 99% in stomach, duodenum and colon and that of NOS in HAP1-ir neurons were 25 - 28% in stomach, duodenum and colon (Fig. 8a-i, Table 2). However, comparatively smaller-sized NOS-ir neurons did not co-express with HAP1 (Fig. 8). Our cell counting data indicated that the co-expression ratio of HAP1 in VIP-ir neurons were 100% in stomach, duodenum and colon and that of VIP in HAP1-ir neurons were 22 - 26% in stomach, duodenum and colon (Fig. 9a-i, Table 2).

Next, we examined the co-expression ratio of HAP1 with ChAT. Our current cell counting data demonstrated that the co-expression ratio of HAP1 in ChAT-ir neurons were 100% in stomach, 69 - 72% in duodenum and colon and that of ChAT in HAP1-ir neurons were 69% in stomach and 60 - 63% in duodenum and colon (Fig. 10 a-i, Table 2). On the other hand, our cell counting data also indicated that the co-expression ratio of HAP1 in SP-ir neurons were 100% in stomach, duodenum and colon and that of SP in HAP1-ir neurons were 26 - 29% in stomach, duodenum and colon (Fig. 11a-i, Table 2).

4.4.3 Immunohistochemical relationships of STB/HAP1 with interneurons and sympathetic noradrenergic neurons

Somatostatin is expressed in descending interneurons in the myenteric plexus (Qu et al. 2008). To examine the immunohistochemical relationship of HAP1 with

interneurons in myenteric plexus, we conducted double-label immunofluorescence histochemistry for HAP1 with somatostatin. Our current cell counting data raveled that the co-expression ratio of HAP1 in somatostatin-ir neurons were 100% in stomach, duodenum and colon and that of somatostatin in HAP1-ir neurons were 2 - 4% in stomach, duodenum and colon (Fig. 12a-i, Table 2).

Next, double staining with antibody TH was done in stomach, duodenum and colon to characterize the sympathetic noradrenergic neurons in myenteric plexus. Our current results indicated that the co-expression ratio of HAP1 in TH-ir neurons were 100% in stomach, duodenum and colon and that of TH in HAP1-ir neurons were 0.17 - 0.27% in stomach, duodenum and colon (Fig. 12j-r, Table 2).

4.4.4 Immunohistochemical relationships of STB/HAP1 with sensory neurons

CGRP has been recognized as well-known marker for intrinsic primary afferent neurons (IPANs)/ sensory neurons (Furness et al. 2004). In this study, CGRP-ir cell bodies were not found in the stomach except some nerve fibers (Fig. 13a-c). In contrast, in the CGRP-ir cell bodies were clearly seen in duodenum and colon (Fig. 13d-i). These results were consistent with previous studies (Mawe et al. 1989; Sayegh and Ritter 2003). Our current double-immunofluorescence results revealed an intriguing result that the co-expression ratio of HAP1 in CGRP-ir neurons were 6 - 9% in duodenum and colon and that of CGRP in HAP1-ir neurons were 2 - 3% in duodenum and colon (Fig.13a-i).

4.5 Discussion

In the current study we have examined the expression of HAP1 in ENS throughout the gastrointestinal tract (esophagus to rectum). We have also determined the

neurochemical phenotypes of HAP1-ir cells in myenteric plexus in stomach, duodenum and colon. The current study is the first to investigate the expression, distribution, and immunohistochemical characterization of HAP1 in the ENS. Although the expression of HAP1 has previously been extensively analyzed in the brain and spinal cord of CNS or dorsal root ganglion in peripheral nervous system previously in a number of studies (Fujinaga et al. 2004, 2007; Sheng et al. 2006; Islam et al. 2012, 2017, 2020a; Wroblewski et al. 2018; Chen et al. 2020; Li et al. 1996; Gutekunst et al. 1998; Dragatsis and Zeitlin 2004; Sheng et al. 2006, 2008; Lin et al. 2010; Niu et al. 2011), the expression of HAP1 or its neurochemical characterization has never been studied in the ENS. Our Western blotting showed that both the isoforms of HAP1A and HAP1B were present in the ENS of adult mouse. In addition, our current immunohistochemical results enunciated that HAP1-immunoreactivity was present in both myenteric and submucosal plexuses of ENS. Our double staining of HAP1 with NeuN confirmed the presence of HAP1-immunoreactivity in neuron of ENS, whereas our current double staining for HAP1 with GFAP, Iba1 or C-Kit ruled out the possibility of the presence of HAP1-immunoreactivity in glial cells or in ICC in ENS. Most of HAP1-ir neurons in ENS had dot-like STB in their cytoplasm, however few neurons had only diffuse HAP1-staining in their cytoplasm with undetectable STBs, as previously observed in the spinal cord or in the brain (Li et al. 1998; Gutekunst et al. 1998; Fujinaga et al. 2009; Islam et al. 2017). Usually HAP1-ir neurons express both HAP1A and HAP1B and the neurons having comparatively more HAP1A show STB in their cytoplasm while those having more HAP1B display only diffuse HAP1-immunoreactivity in their cytoplasm (Fujinaga et al. 2007; Islam et al. 2017, 2020a; Wroblewski et al. 2018). Our current results elucidate that in addition to the CNS, STB/HAP1-immunoreactivity is also abundantly expressed in the ENS. The most striking result in the present study is

that a very high percentage of the myenteric motor neurons (both excitatory and inhibitory), and interneurons express HAP1-immunoreactivity, while the IPANS are almost devoid of HAP1-immunoreactivity in myenteric neurons across different segments of the ENS (summarized in Fig. 14). Our current findings may reflect HAP1′s involvement in modulating certain functions of motor neurons or interneurons in myenteric plexus.

4.5.1 Both inhibitory and excitatory motor neurons express HAP1

To characterize the neurochemical phenotypes of HAP1, we used the markers for two major transmitters expressed in myenteric neurons, NOS, the enzyme catalyzing the production of nitric oxide, and ChAT, the synthesizing enzyme for acetylcholine. Nitric oxide and acetylcholine are the key neurotransmitters regulating inhibitory and excitatory inputs, respectively (Brookes 2001; Mazzoni et al. 2020). More than 95% of the myenteric neurons are positive for either NOS or ChAT, whereas less than 5% neurons are positive for both, and the remaining very small number of neurons express neither ChAT nor NOS (Murphy et al. 2007; Porter et al. 2002; Wattchow et al. 2008). NOS-immunoreactivity are largely limited to Dogiel type I neurons (Qu et al. 2008). In addition to the NOS, the inhibitory neurons also express VIP (Furness 2000). The majority of ChAT-ir excitatory myenteric nerve cell bodies are Dogiel type I, however, there is also a small number of large pale-staining ChAT-ir Dogiel type II neurons (Sang 1998). One of the striking findings in our current study was that about 97-99% of NOS-ir cells and 100% of VIP-ir cells contained HAP1-immunoreactivity. These results suggest that almost all the inhibitory neurons contained HAP1 in myenteric plexus. Another striking finding in our current study was that about 100% of ChAT-ir cells in stomach or 69-72% of ChAT-ir cells in intestine contained HAP1-immunoreactivity. It

has been reported that a large number of the ChAT-ir neurons contained SP and calcium-binding proteins (Sang 1998). In the present study we also performed double staining of HAP1with SP or calcium binding proteins (calbindin, calretinin). We found an intriguing result that 100% SP-ir neurons and a large number of calbindin- or calretinin-ir neurons expressed HAP1. Taken together, our current results reveal that excitatory neurons also contain HAP1.Taking the methodological limitations into account, our current results may indicate that almost all the excitatory and inhibitory motor neurons contain HAP1-immunoreactivity in myenteric plexus. HAP1 could be considered as a potential neurochemical marker for both excitatory and inhibitory neuron groups in the ENS.

4.5.2 All the interneuron and catecholaminergic neurons contain HAP1 but sensory neuron shows lack of HAP1-immunoreactivity

There are ascending (orally directed) and descending (anally directed) types of interneurons in the ENS (Brehmer et al. 1999; Furness 2000). The ascending neurons are mostly cholinergic (Kunze and Furness 1999), whereas the descending types of interneurons show different chemical coding: ChAT/NOS/VIP, ChAT/SOM or ChAT/5-HT (Mazzoni et al. 2020; Furness 2000). It has been hypothesized that ChAT/NOS/VIP neurons regulate local motility reflexes, ChAT/SOM neurons modulate conduction of migrating myoelectric complexes, and the ChAT/5-HT modify secretomotor reflexes (Pompolo and Furness 1998; Furness 2000). As our current study shows that almost all the ChAT-ir neurons contain HAP1-immunoreactivity, it is highly possible that ascending types of interneurons also express HAP1. To answer the question whether the descending types of interneurons contain HAP1-immunoreactivity, we double-stained HAP1 with somatostatin. Intriguingly, our

current results confirmed that 100% of somatostatin neurons also contained HAP1. These results clearly indicate that both ascending and descending types of interneurons contain HAP1-immunoreactivity.

In the present study, we also performed double-staining of HAP1 with TH to further clarify the phenotype of HAP1-ir cells in myenteric plexus. TH-immunoreactivity occurs in a very small proportion of myenteric neurons, which are usually Dogiel type I in morphology (Qu et al. 2008). The role of TH-ir neurons is not well defined. One group explained the TH-ir neurons as dopaminergic (Li et al. 2004), whereas other group described as noradrenergic extrinsic sympathetic neurons (Anlauf et al. 2003). In a broader term, the phenotype of TH-ir neurons are considered as catecholaminergic neurons (Noorian et al. 2011). The present study confirmed that 100% of TH neurons expressed HAP1-immunoreactivity, suggesting that the catecholaminergic neurons in myenteric plexus also contained HAP1.

Finally, we examined the immunohistochemical relationships of HAP1 with IPANs (sensory neurons). Morphologically, the IPANs are Dogiel type II neurons (Furness 2000). The characteristics electrophysiological properties of these Dogiel type II neurons discerns them from motor neurons and interneurons (Furness et al. 1998). CGRP is a good chemical marker of Dogiel type II IPANs (Qu et al. 2008). Studies on the effects of CGRP receptor antagonists during reflex activation clarifies that IPANs in mouse intestine contain and release CGRP. Almost all the CGRP-ir neurons have similar morphological appearance as that to Dogiel type II cells. However, there are also a very few CGRP- ir Dogiel type I neurons (Qu et al. 2008). In the present study, we have found CGRP-ir neurons in different segments of gastrointestinal tract except

in stomach. We detected only CGRP-ir nerve fibers in the stomach. These findings were similar with that of previous studies (Mawe et al. 1989; Sayegh and Ritter 2003), where they also found only CGRP-ir nerve fibers in stomach and suggested that those fibers were probably extrinsic in origin. Our current results provide intriguing evidence that HAP1 never co-localize with large, oval, or bigger-sized CGRP-ir neurons (Dogiel type II), whereas a very few small-sized CGRP-ir neurons (possibly Dogiel type I) were HAP1 positive. Although a detailed morphological or electron microscopical studies are needed in future, our current results may suggest that the typical Dogiel type II neurons are almost devoid of HAP1-immunoreactivity.

4.5.3 Possible physiological role of HAP1 in ENS

Our current study clearly showed that HAP1 is highly co-expressed with the markers of motor neurons (excitatory or inhibitory), interneurons, and catecholaminergic extrinsic sympathetic neurons but not with that of sensory neurons in myenteric plexus. Motor neurons and interneurons include Dogiel type I neurons, whereas sensory neurons contain Dogiel type II neurons (Lomax and Furness 2000; Brookes 2001). Our current findings indicate that HAP1-immunoreactivity selectively present in Dogiel type I neurons. Although the gastrointestinal tract receives input from CNS through the vagus nerve and thoracolumbar/lumbosacral spinal ganglion, ENS also can act independently of the CNS (Fleming et al. 2020; Fung and Vanden Berghe 2020). In the myenteric plexus, inhibitory and excitatory motor neurons project fibers to the longitudinal and circular muscles (Furness 2000; Fung and Vanden Berghe 2020). Excitatory motor neurons project orally and use acetylcholinesterase as their primary transmitter, whereas inhibitory motor neurons project anally and utilize NOS or VIP. The excitatory motor neurons stimulate smooth muscle contraction and the inhibitory

motor neurons induce smooth muscle relaxation (Fleming et al. 2020). There are two main types of interneurons located in the myenteric plexus: ascending or orally directed interneurons and descending or anally directed interneurons (Brehmer et al. 1999, Furness 2000). These interneurons have been noted to form chains that extend the length of the GI tract and believed to involve in the migrating myoelectric complex of the small intestine (Kunze and Furness 1999). Abundant expression of HAP1-immunoreactivity in the excitatory motor neuron, inhibitory motor neuron, and in interneurons (both ascending and descending) might imply a vital role of HAP1 in regulating physiological functions of these neurons, including bowel motility control, blood flow to permit nutrient and fluid absorption, secretion, and support waste elimination (Avetisyan et al. 2015). Detailed morphological and physiological experiments are needed in future to elucidate the effects of HAP1 in the myenteric neurons.

4.5.4 Possible pathological role of HAP1 in ENS

It has been reported that STB/HAP1 can increase the threshold of vulnerability to neurodegeneration and provide increased stability to neurons expressing HAP1, and subsequently protect neurons against apoptosis/cell death. In our previous studies, we have showed that HAP1 can sequester the pathological mutant molecules and trap the toxic aggregation in the cytoplasm to prevent apoptosis-inducing nuclear translocation (Takeshita et al. 2006, 2011). We have also reported that the neurodegeneration-prone regions, including cortex, thalamus, striatum, cerebellum, and motoneurons are devoid of STB/HAP1-expression, whereas the regions relatively spared from neurodegeneration, such as preoptic area, hypothalamic area or spinal dorsal horn are rich in STB/HAP1-expression (Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Islam

et al. 2012, 2017). According to our current results, it can be speculated that HAP1-immunoreactivity might increase the threshold of vulnerability to neurodegeneration or give out beneficial stability to myenteric motor neuron and interneuron but not to IPAN.

In a recent study, it has been shown that the expression of HAP1 is significantly reduced in pancreatic cancer (Li et al. 2019). HAP1-expression is also decreased in breast cancer tissues and overexpression of HAP1 reduced the cell proliferation in breast cancer (Zhu et al. 2013). These studies suggested that HAP1 may serve as a potential diagnostic marker for certain cancer. Our current results may also provoke to investigate the expression of STB/ HAP1-protection in the enteric neurons throughout the gastrointestinal tract of different neurodegenerative disease mouse model. The characterization of HAP1 in enteric nervous system is very pivotal for further experiment of genetically engineered, mutant or transient mice in order to enhance the prospect of digestive degenerative disorders (Van der Burg et al. 2009; Sassone et al. 2009). Mutant hungtintin (htt), one of the causal agents of Huntington disease not only present in the CNS but also aggreate in the non-CNS tissues (Moffitt et al. 2009; Sathasivam et al. 1999). It has been known that the effects of neurodegenerative diseases (such as Huntington's disease, Parkinson's disease, and Alzheimer's disease) have been recognized in ENS in addition to CNS (Björkqvist et al. 2008; Chaumette et al. 2009; Lebouvier et al. 2009; Khan and Alkon 2010). Noteworthy, HD patients show gastrointestinal dysfunction and have several sign & symptoms including loss of enteric neuropeptides, decreased mucosal thickness and villus length as well as impaired gut motility (Van der Burg et al. 2009). Other problems such as loss of body weight (Djousse et al. 2002; Farrer and Yu, 1985; Robbins et al.

2006; Sanberg et al. 1981), malnutrition (Lanska et al. 1988), difficulties in swallowing (Wood et al. 2008), upper gastrointestinal dysfunction like gastritis and esophagitis(Andrich et al. 2009) have also been observed. The unintended loss of body weight have been found mainly in the three major types of neurodegenerative diseases: Alzheimer's disease, Parkinson's disease and Huntington's disease (Aziz et al. 2008). The role of HAP1/STB might have some resistance mechanism in the pathological pathways in the ENS to prevent the extended number of CAG repeats by maintaining the threshold numbers in the huntingtin gene. It is intriguing to consider that the downregulated STB/HAP1-expression in ENS could be a potential risk factor for specific neurodegenerative disorders. However, no direct observation of this hypothesis has been known to date, and the link between STB/HAP1-expression and enteric neuropathology could be an active area of future research. Furthermore, Hirschsprung disease is a fatal birth defect in which the enteric neurons are missing from the bowel (Heuckeroth 2018), whereas, achalasia is a motility disorder of the esophagus, which results in degeneration of the myenteric plexus of the esophageal wall (O'Neill et al. 2013). Future studies should also include detailed *in vitro* and *in vivo* studies to clarify the effects of HAP1 on the vulnerability of enteric neurons regarding these disorders.

Mounting evidence suggests that healthy gut with diverse microbes is vital for normal brain functions (Sharon et al. 2016; Suganya and Koo 2020). Changes in the bidirectional relationship between the gastrointestinal tract and CNS are linked with the pathogenesis of gastrointestinal and neurological disorders. Therefore, the microbiota/gut-brain axis is an emerging and widely accepted concept (Sharon et al. 2016; Suganya and Koo 2020; Kuwahara et al. 2020). The gut microbiota affects

several physiological processes, including gut motility (Carabotti et al. 2015). Further, alteration of gut microbiota composition can influence the cognition, sleep, and mood disorders (Cryan and Dinan 2012). In addition, research over past few years elucidates that the gut microbiota plays a vital role in the pathogenesis of neurodegenerative diseases, including Parkinson's diseases, Alzheimer diseases, multiple sclerosis and Huntington's disease (Sharon et al. 2016; Castillo-Álvarez and Marzo-Sola 2019; Wasser et al. 2020). It has been reported that IPANs project sensory nerve fibers to the gut mucosa. IPANs also project nerve fibers directly to interneurons and motor neurons through which they send chemical signals from gut microbiota or microbiota metabolites to other enteric neurons or to the CNS (Kuwahara et al. 2020). According to our present results, it is tempting to speculate that due to lack of STB/HAP1 protectivity IPANs-related gut sensory system might be more vulnerable to stress conditions than STB/HAP1-expressing gut motor system. Intriguingly, the incidence of certain gastrointestinal disorders increaseas with the advancement of age and paralelly the enteric neurons also degenerate with age (Britton and McLaughin 2013; Phillips and Poweley 2007). Interestingly, expression of HAP1 mRNA in the neurons of CNS is also downregulated in aged rodents (Page et al. 1998). Although further studies need to be performed to examine the age related changes in HAP1 expression in the ENS, it is compelling to speculate that the lower level of HAP1-expression might be responsible for gastrointestinal disorders vis-à-vis neurodegeneation in the ENS/CNS in elderly populations. Further detailed studies are required to elucidate the effects of STB/HAP1 in protection of microbiota/gut-brain axis.

In conclusion, our present study is the first to clarify the expression HAP1 in the ENS. The present study is also the first to elucidate the neurochemical phenotypes

of HAP1-ir cells in myenteric plexus. Our current result shows that HAP1 is highly expressed in the ENS throughout the gastrointestinal tract. HAP1-immunoreactivity is present in excitatory motor neurons, inhibitory motor neurons, and interneurons of myenteric plexus. Our current results may suggest the importance of HAP1 in regulation of both excitatory and inhibitory motor neuron or interneuron functions. HAP1 could be used as a potential novel neurochemical marker for Dogiel type I neurons. It will be of great interest to elucidate the pathophysiological roles of HAP1 in ENS. Due to lack of putative STB/HAP1 protectivity, Dogiel type II neurons might be more vulnerable to neurodegeneration than STB/HAP1-expressing Dogiel type I neurons.

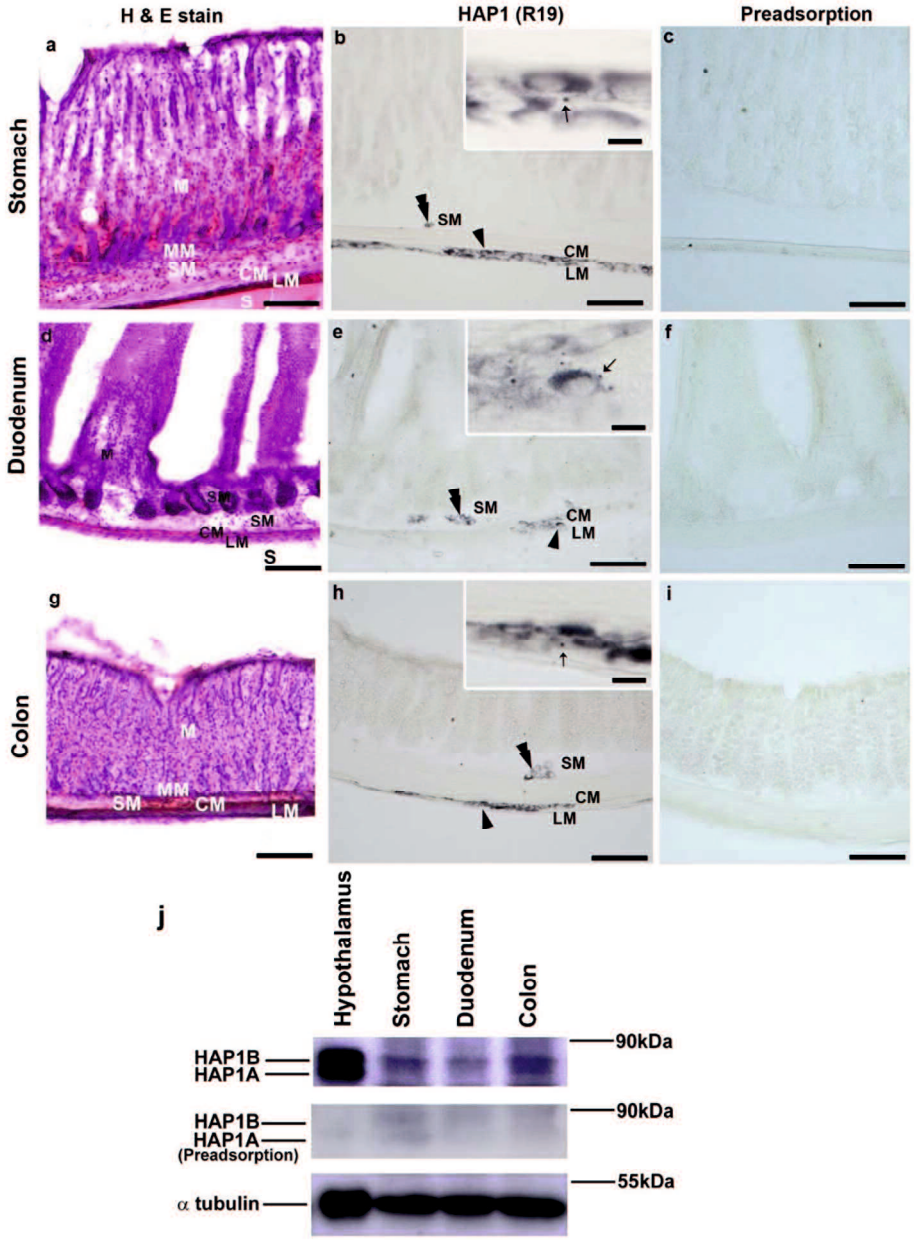

Fig. 1 Immunohistochemistry for huntingtin-associated protein 1 (HAP1) in tissue sections and Western blotting. Photomicrographs showing H & E stain (a, d, g), expression of HAP1-ir cells (b, e, h) in the myenteric plexus and pre-adsorption control test (c, f, i) in stomach, duodenum and colon and western blotting for huntingtin-associated protein 1 (HAP1) (j). Location of serosa (S), longitudinal muscle (LM), circular muscle (CM), submucosa (SM), muscularis mucosae (MM) and mucosa (M) are indicated in H & E stain. HAP1-immunoreactivty was expressed in the myenteric plexus (between CM and LM) and in the submucosal plexus in SM. Insets are the enlargement of HAP1-ir cells indicated by arrowhead in myenteric plexus. Arrow indicates the HAP1-ir stigmoid body (STB). Double arrowhead indicates the HAP1-ir cells in the submucosal plexus. Preincubation with a blocking peptide against the HAP1 primary antibody resulted in disappearance of the HAP1-immunoreactivity (c, f, i). Western blot analysis (j) using lysate from the hypothalamus (control) and different segment of gastrointestinal tract (stomach, duodenum, colon) showing bands of approximately 85 kDa for HAP1B, 75 kDa for HAP1A using HAP1 antibody, whereas with a blocking peptide against HAP1 antibody resulted in eliminating of the HAP1-positive bands. Scale bar =100μm (a-i) and 10μm in insets

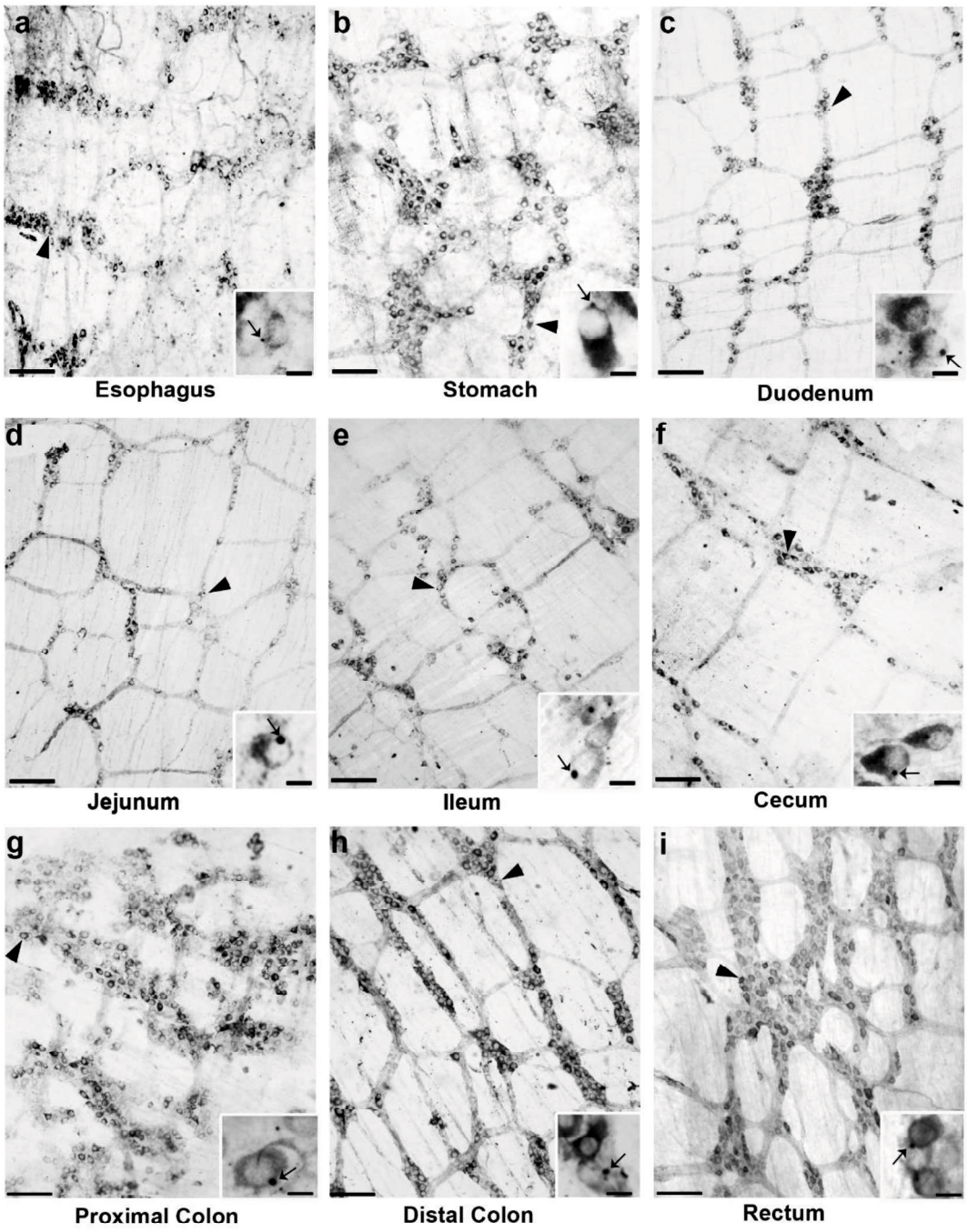

Fig. 2 Single immunoperoxidase staining of huntingtin-associated protein 1 (HAP1) in whole mount preparations. Photomicrographs showing HAP1-ir cells in the myenteric plexus from esophagus to rectum (a-i) in whole mount preparations. Insets are the enlargement of HAP1-ir cells indicated by arrowhead and arrow indicate the HAP1-ir stigmoid body (STB). Scale bar =100μm (a-i) and 10μm in insets.

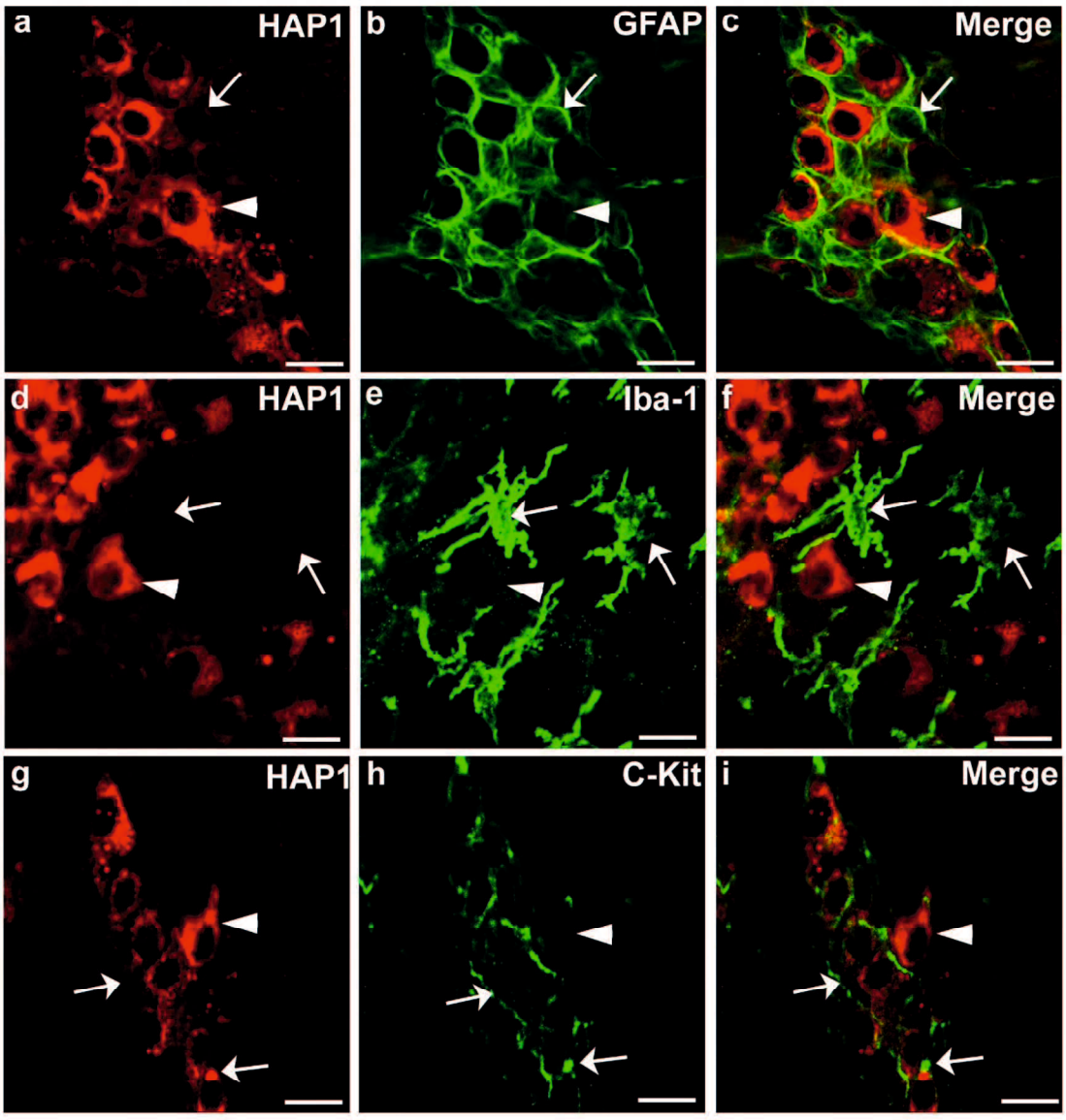

Fig. 3 Double-label immunofluorescence immunohistochemistry for HAP1 with GFAP, Iba-1 and C-kit. Photomicrographs showing immunolabeling of HAP1 with GFAP (a-c), Iba-1(d-f) and c-kit (g-i) in the myenteric plexus of mouse colon in whole mount preparations. Arrowheads indicate the cells single positive for HAP1 and arrows indicate the glial process of GFAP, mascular macrophase (Iba-1) and interstitial cell of cajal (c-kit). Scale bar =20μm (a-i).

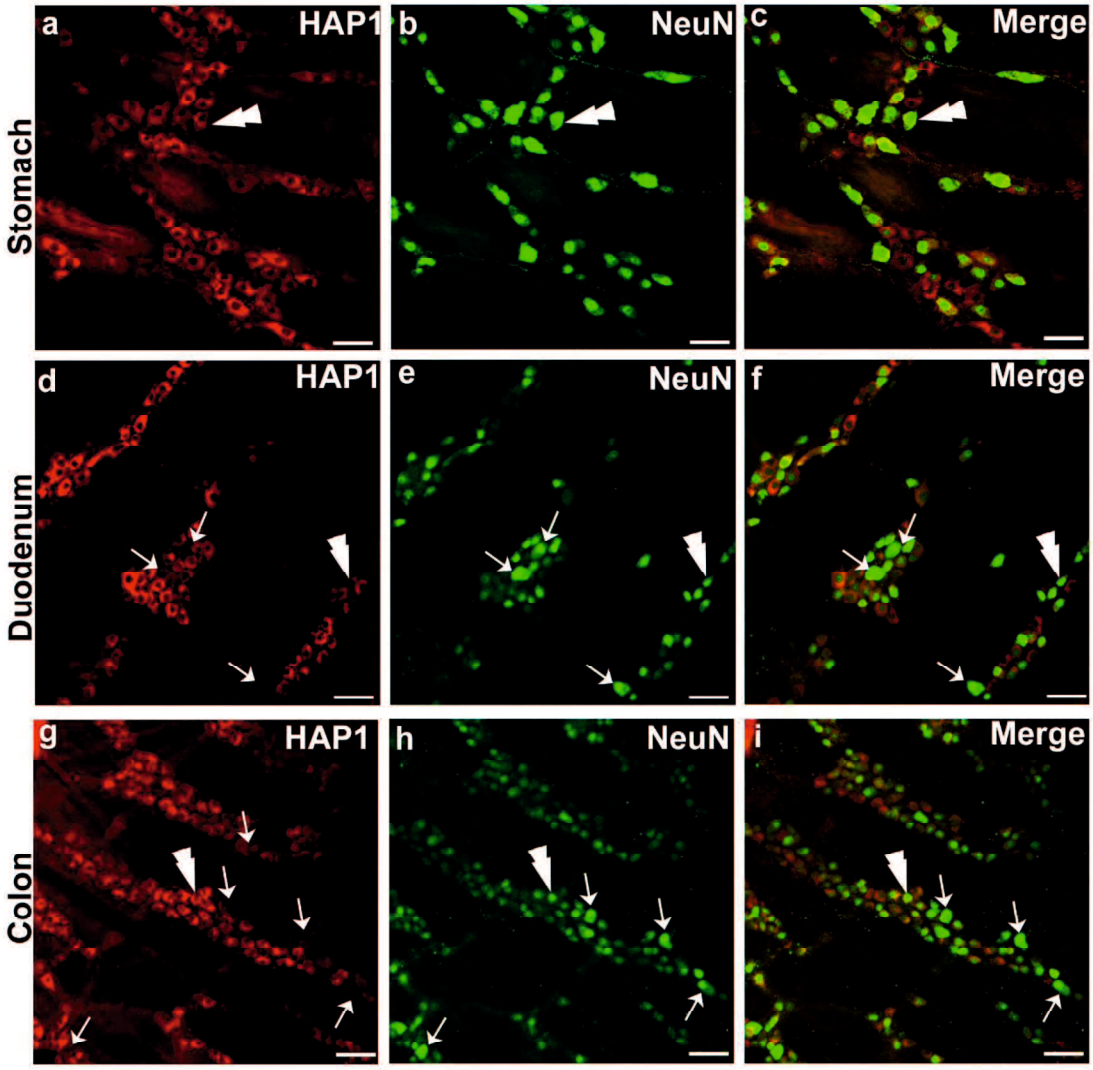

Fig. 4 Double-label immunofluorescence immunohistochemistry for HAP1 with NeuN. Photomicrographs showing immunolabeling of HAP1 with NeuN in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i) in whole mount preparations. Arrowheads indicate the cells single positive for HAP1, double arrowheads indicate the double labeled neurons of HAP1 and NeuN. Arrows indicate the cells single positive for NeuN. Scale bar =50μm (a-i).

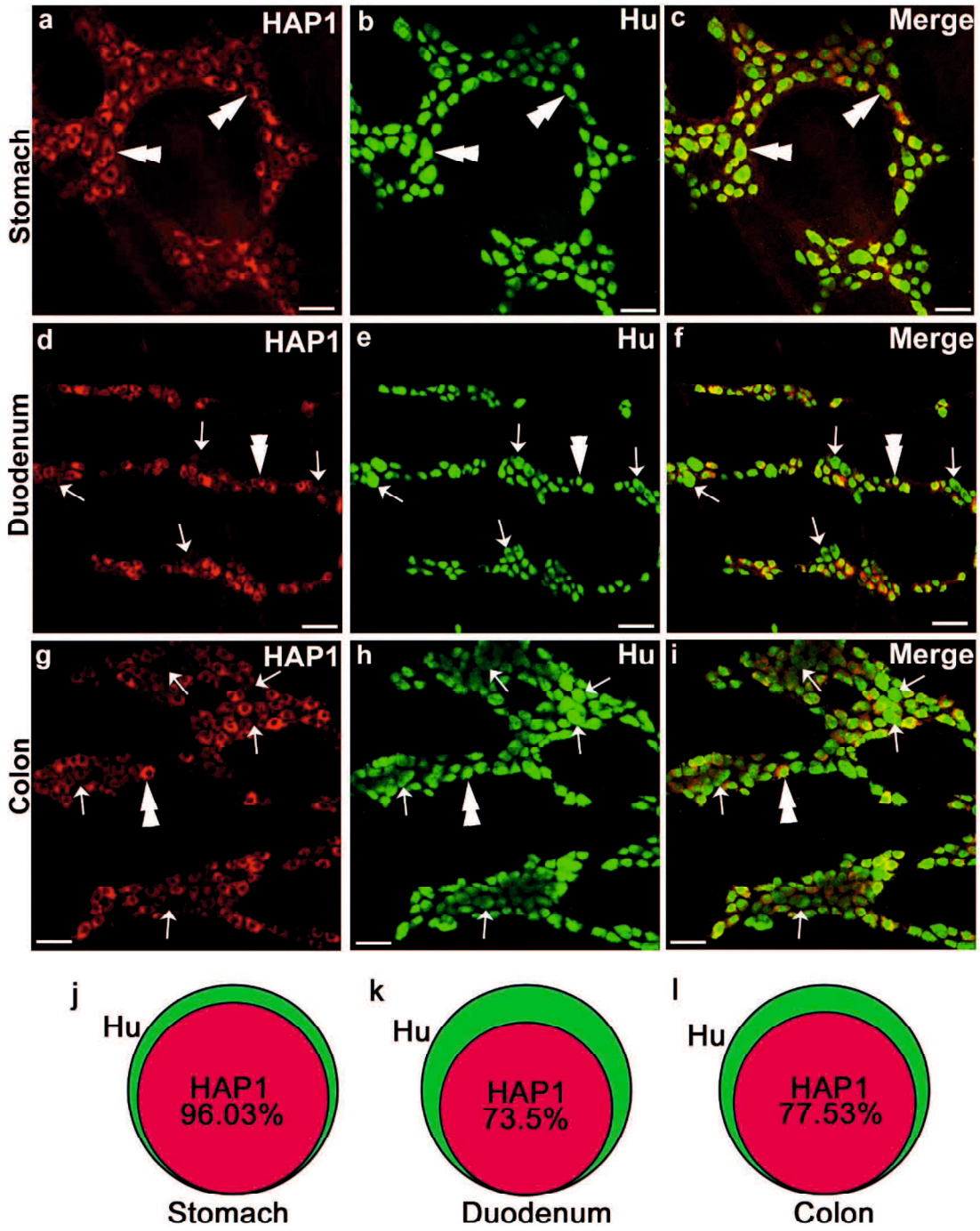

Fig. 5 Double-label immunofluorescence immunohistochemistry for HAP1 with Hu. Photomicrographs showing immunolabeling of HAP1 with Hu in the myenteric plexus of mouse stomach (a-c), duodenum (d- f), colon (g-i) in whole mount preparations. Double arrowheads indicate the double labeled neurons of HAP1 and Hu. Arrows indicate the cells single positive for Hu. Scale bar = 50μm (a-i). Pseudo Venn-diagram indicating the relative extent to which HAP1 express Hu in stomach, duodenum and colon (j-l).

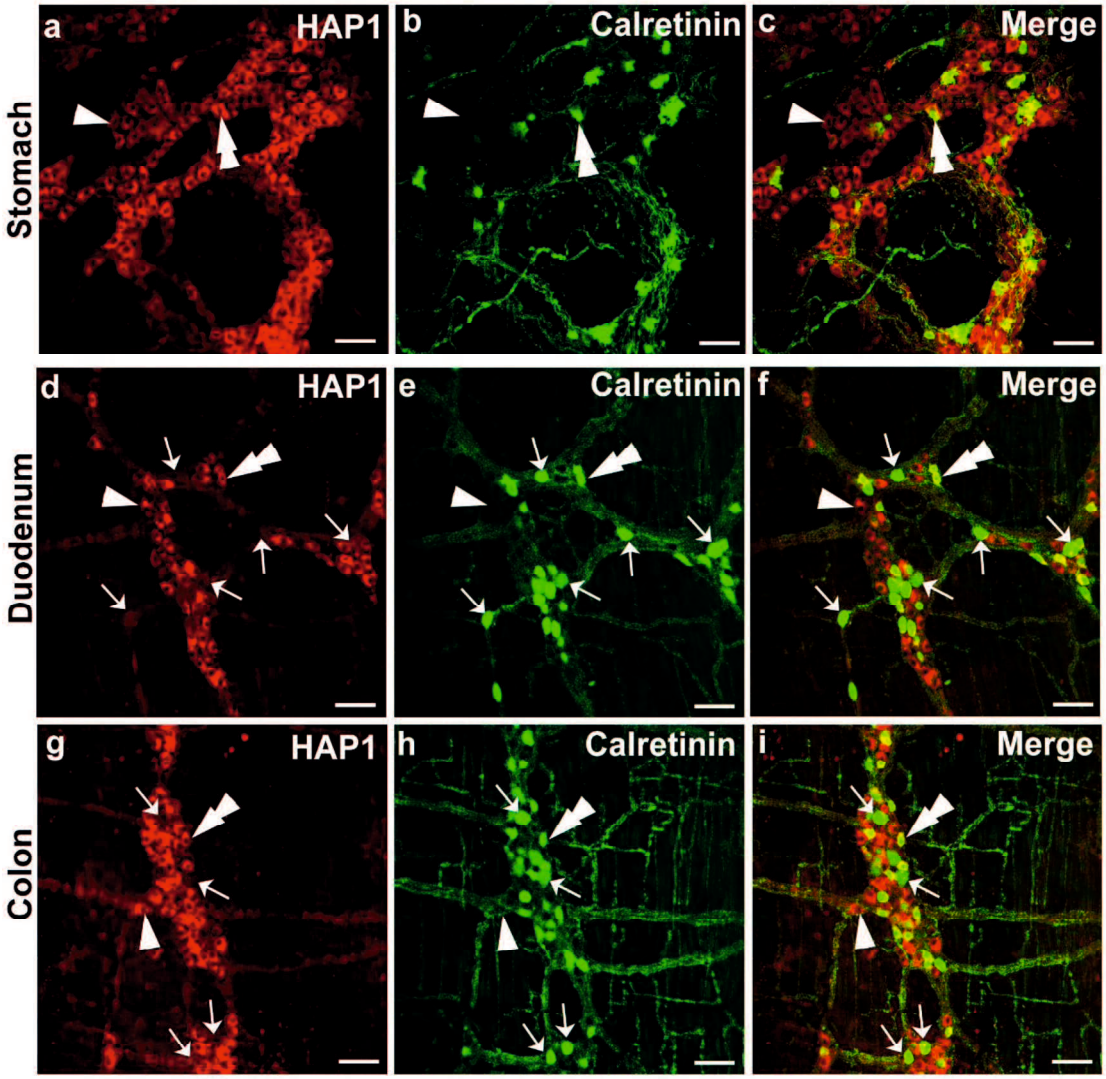

Fig. 6 Double-label immunofluorescence immunohistochemistry for HAP1 with calretinin. Photomicrographs showing immunolabeling of HAP1 with calretinin in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i) in whole mount preparations. Arrowheads indicate the cells single positive for HAP1 and double arrowheads indicate the double labeled neurons of HAP1 and calretinin. Arrows indicate the cells single positive for calretinin. Scale bar =50μm (a-i).

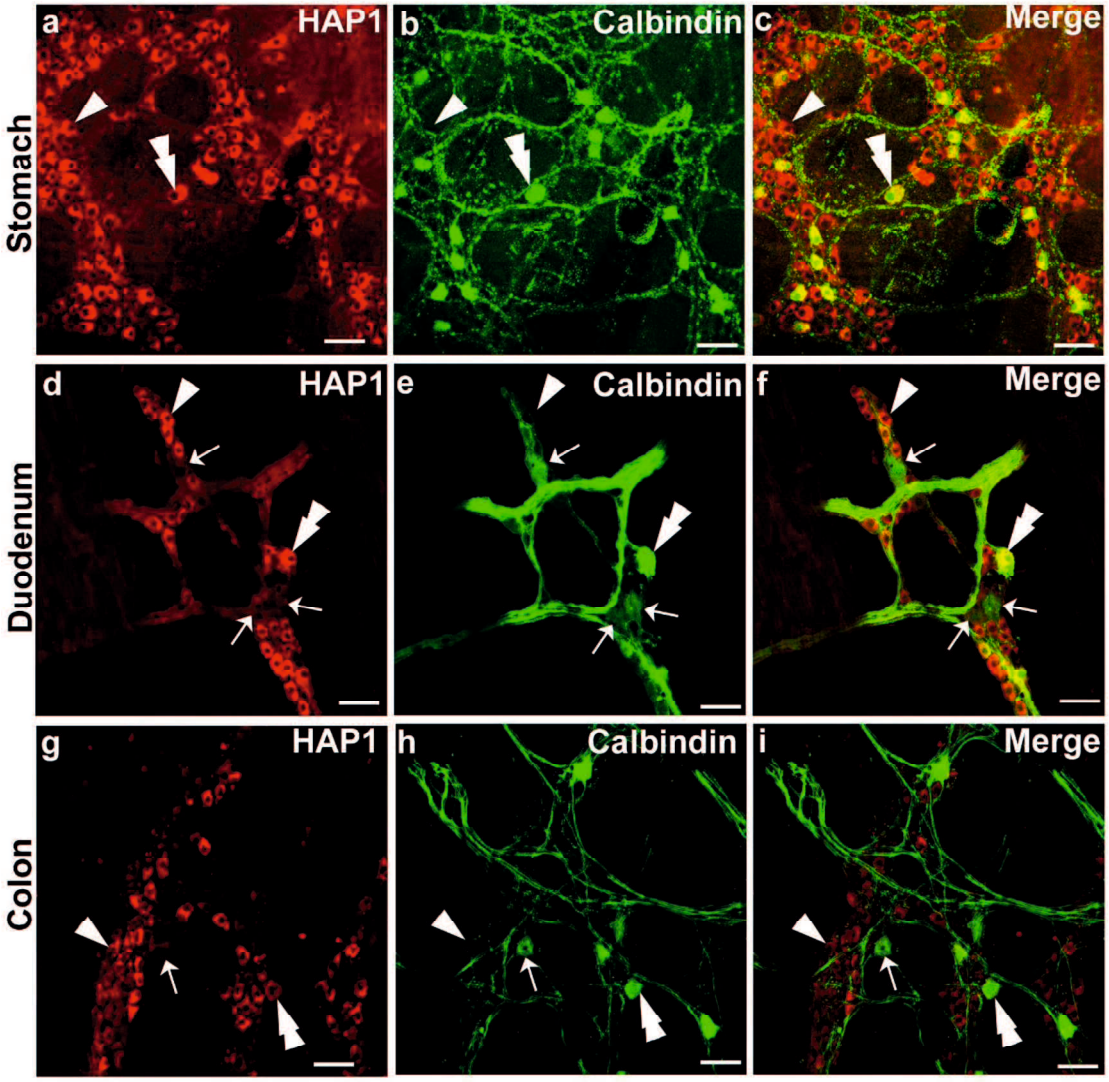

Fig. 7 Double-label immunofluorescence immunohistochemistry for HAP1 with calbindin. Photomicrographs showing immunolabeling of HAP1 with calbindin in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i) in whole mount preparations. Arrowheads indicate the cells single positive for HAP1 and double arrowheads indicate the double labeled neurons of HAP1 and calbindin. Arrows indicate the cells single positive for calbindin. Scale bar =50μm (a-i).

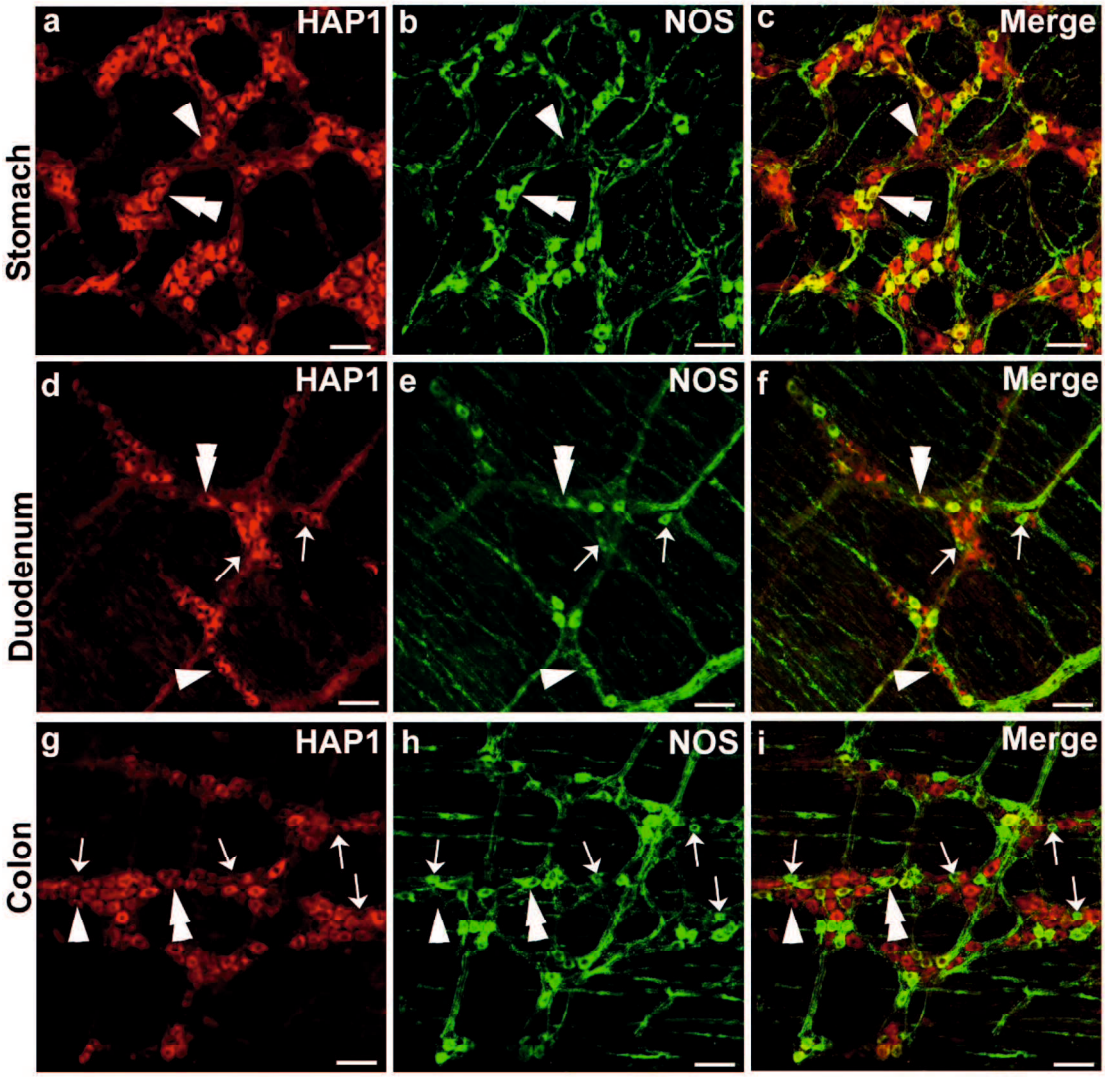

Fig. 8 Double-label immunofluorescence immunohistochemistry for HAP1 with NOS. Photomicrographs showing immunolabeling of HAP1 with NOS in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i) in whole mount preparations. Arrowheads indicate the cells single positive for HAP1 and double arrowheads indicate the double labeled neurons of HAP1 and NOS. Arrows indicate the small size cells single positive for NOS. Scale bar =50μm (a-i).

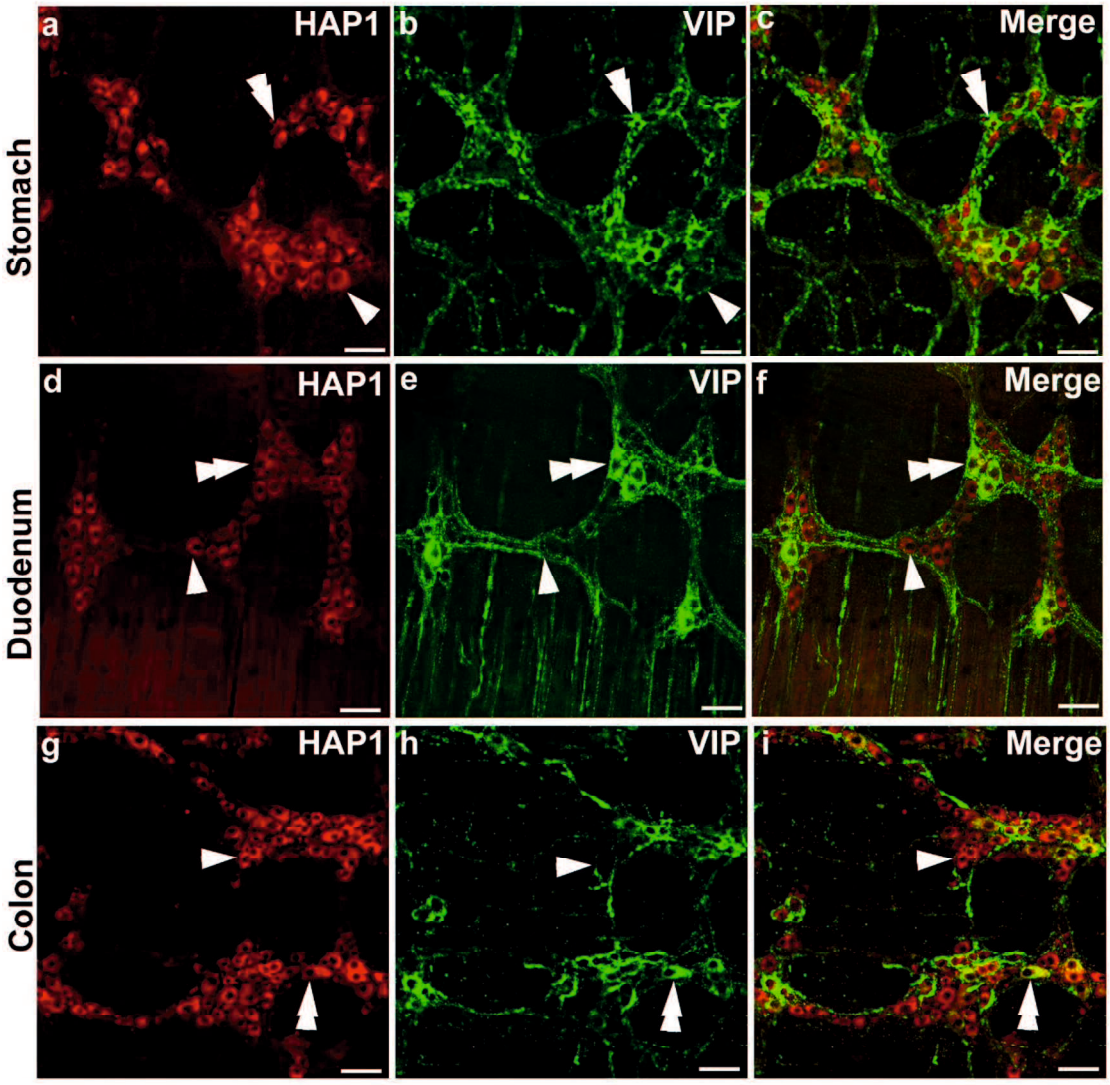

Fig. 9 Double-label immunofluorescence immunohistochemistry for HAP1 with VIP. Photomicrographs showing immunolabeling of HAP1 with VIP in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i) in whole mount preparations. Arrowheads indicate the cells single positive for HAP1 and double arrowheads indicate the double labeled neurons of HAP1 and VIP. Arrows indicate the small size cells single positive for VIP. Scale bar =50μm (a-i).

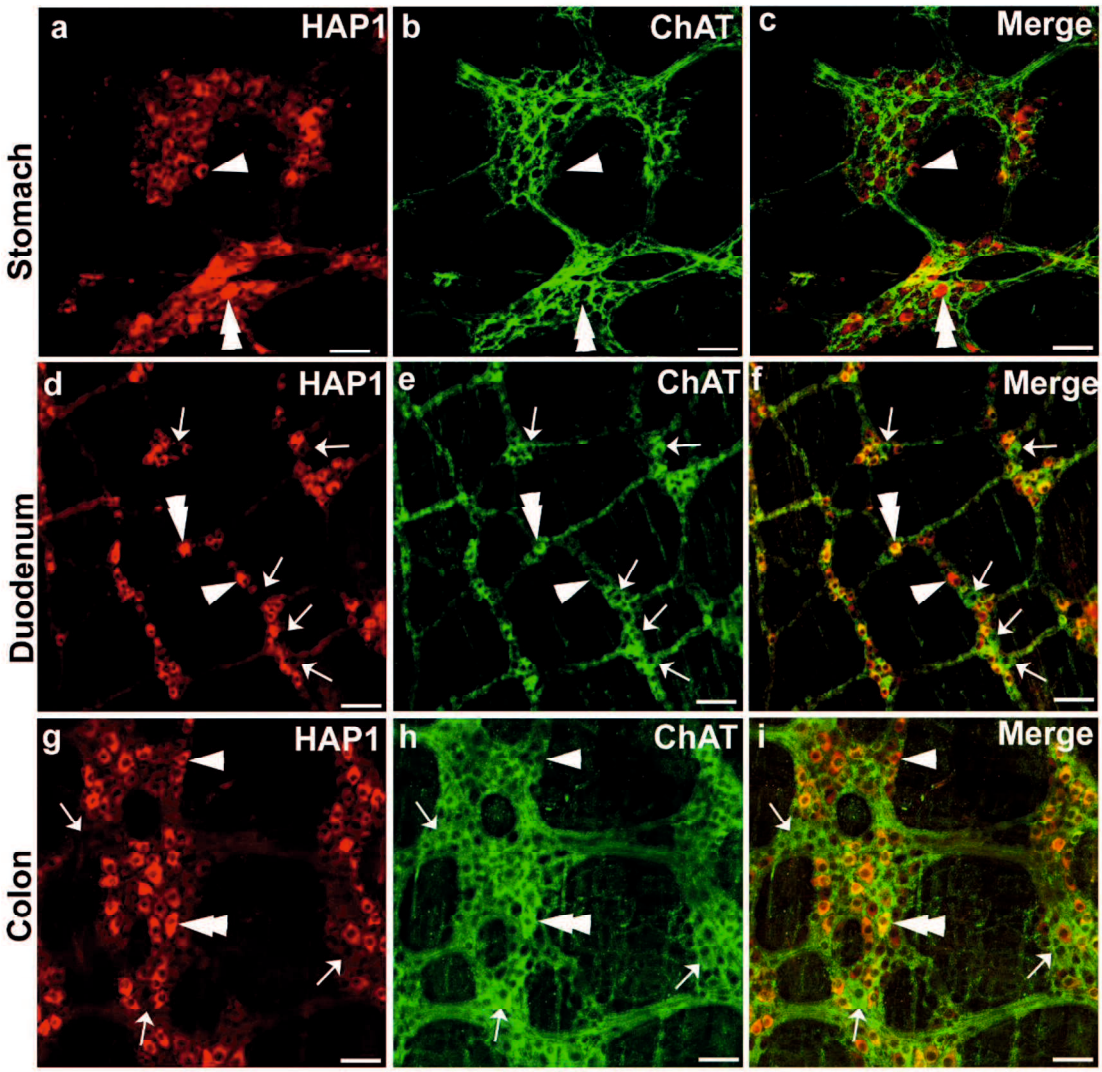

Fig. 10 Double-label immunofluorescence immunohistochemistry for HAP1 with ChAT. Photomicrographs showing immunolabeling of HAP1 with ChAT in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i) in whole mount preparations. Arrowheads indicate the cells single positive for HAP1 and double arrowheads indicate the double labeled neurons of HAP1 and ChAT. Arrows indicate the cells single positive for ChAT. Scale bar =50μm (a-i).

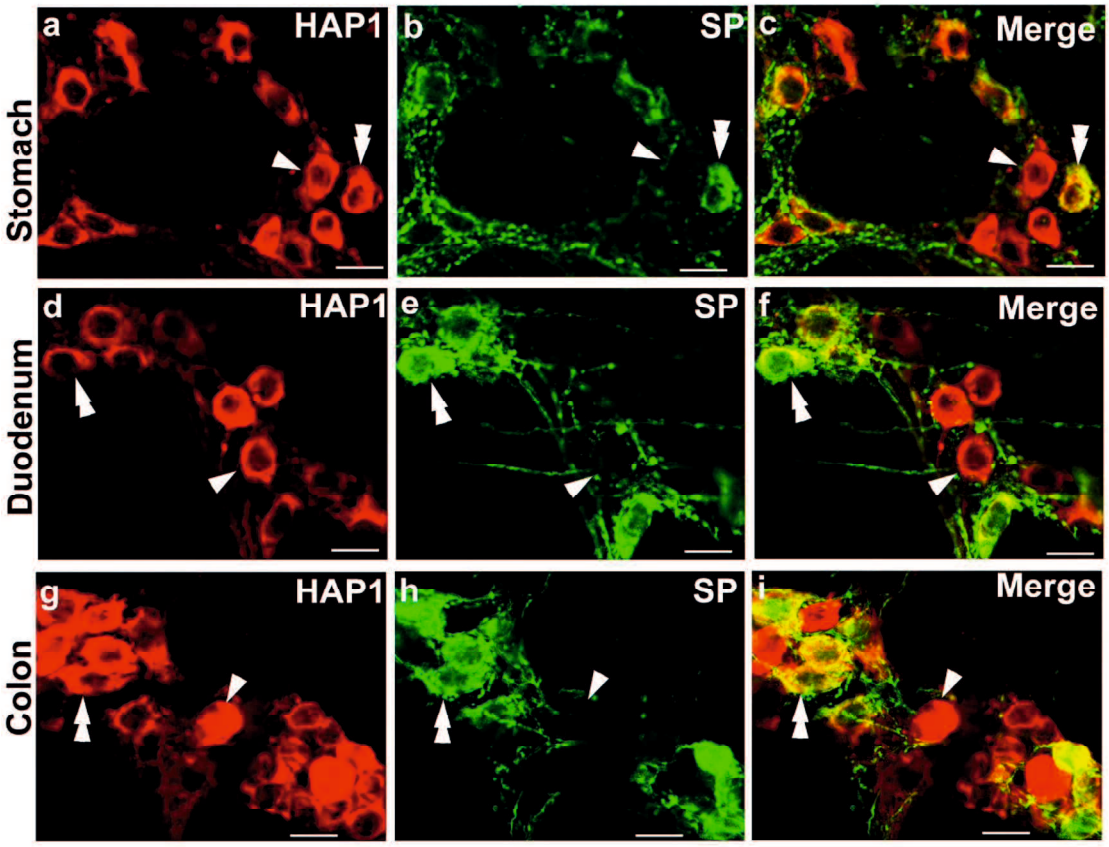

Fig. 11 Double-label immunofluorescence immunohistochemistry for HAP1 with SP. Photomicrographs showing immunolabeling of HAP1 with SP in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i) in whole mount preparations. Arrowheads indicate the cells single positive for HAP1 and double arrowheads indicate the double labeled neurons of HAP1 and SP. Scale bar =20μm (a-i).

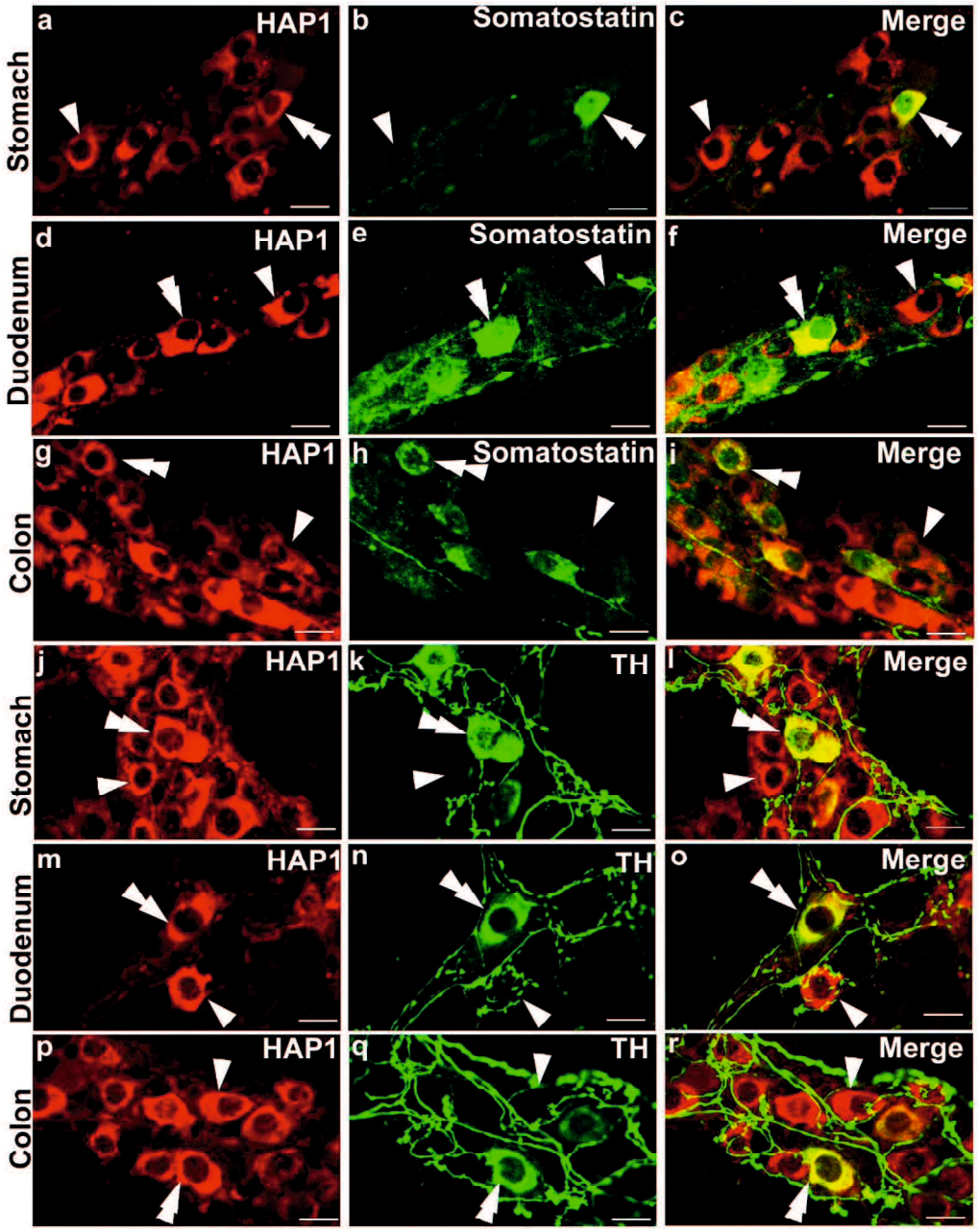

Fig. 12 Double-label immunofluorescence immunohistochemistry for HAP1 with somatostatin and TH. Photomicrographs showing immunolabeling of HAP1 with somatostatin in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i) in whole mount preparations. Photomicrographs also showing HAP1 with TH in stomach (j-l), duodenum (m-o), colon (p-r) in the myenteric plexus of whole mount preparations of mouse. Arrowheads indicate the cells single positive for HAP1 and double arrowheads indicate the double labeled neurons (HAP1 with somatostatin, and HAP1 with TH). Scale bar =20μm (a-r).

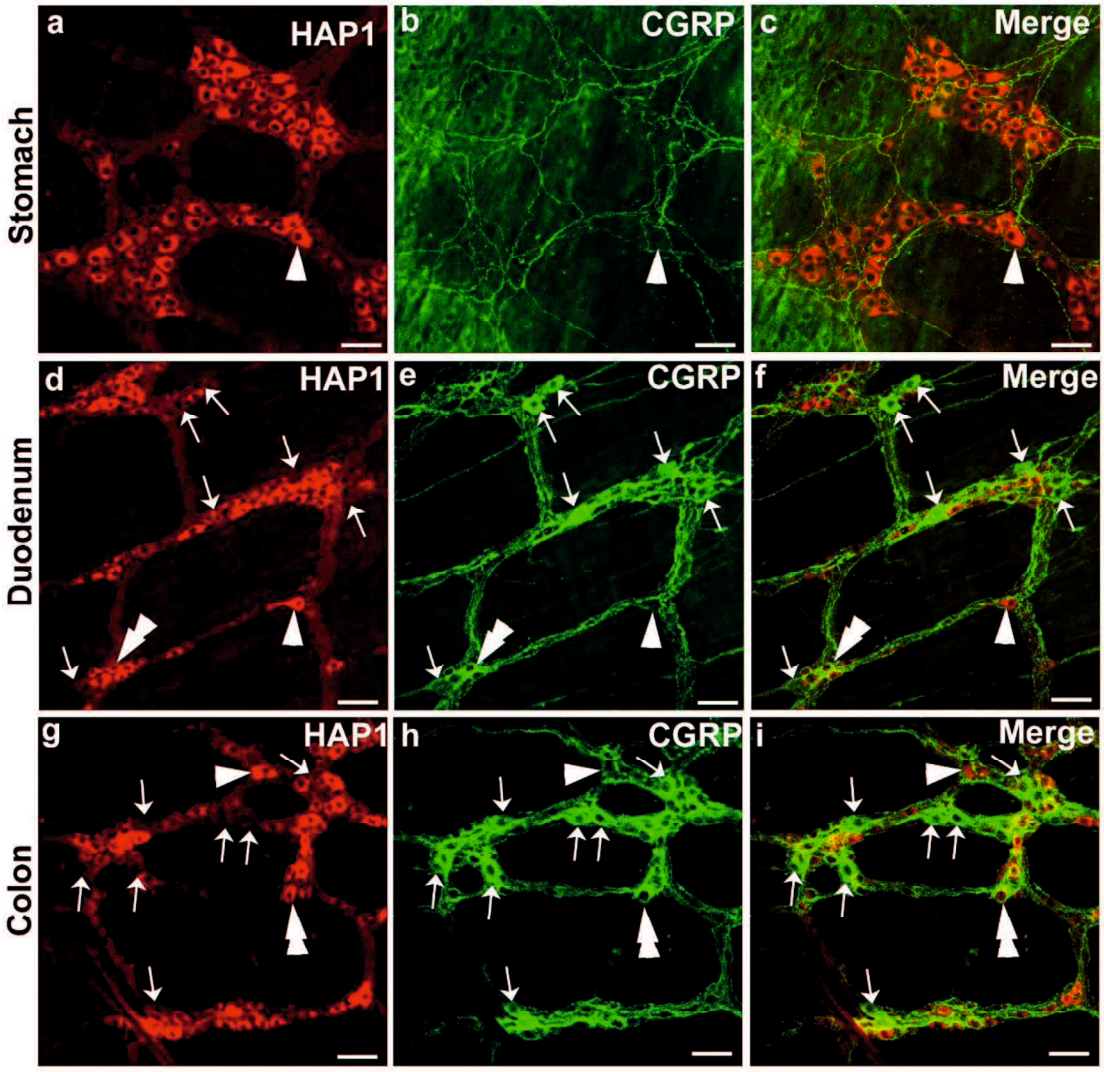

Fig. 13 Double-label immunofluorescence immunohistochemistry for HAP1 with CGRP. Photomicrographs showing immunolabeling of HAP1 with CGRP in the myenteric plexus of mouse stomach (a-c), duodenum (d-f), colon (g-i). Arrowheads indicate the cells single positive for HAP1 and arrows indicate the cells single positive for CGRP. Double arrowheads indicate the double labeled neurons of HAP1 and CGRP. Scale bar =50μm (a-i).

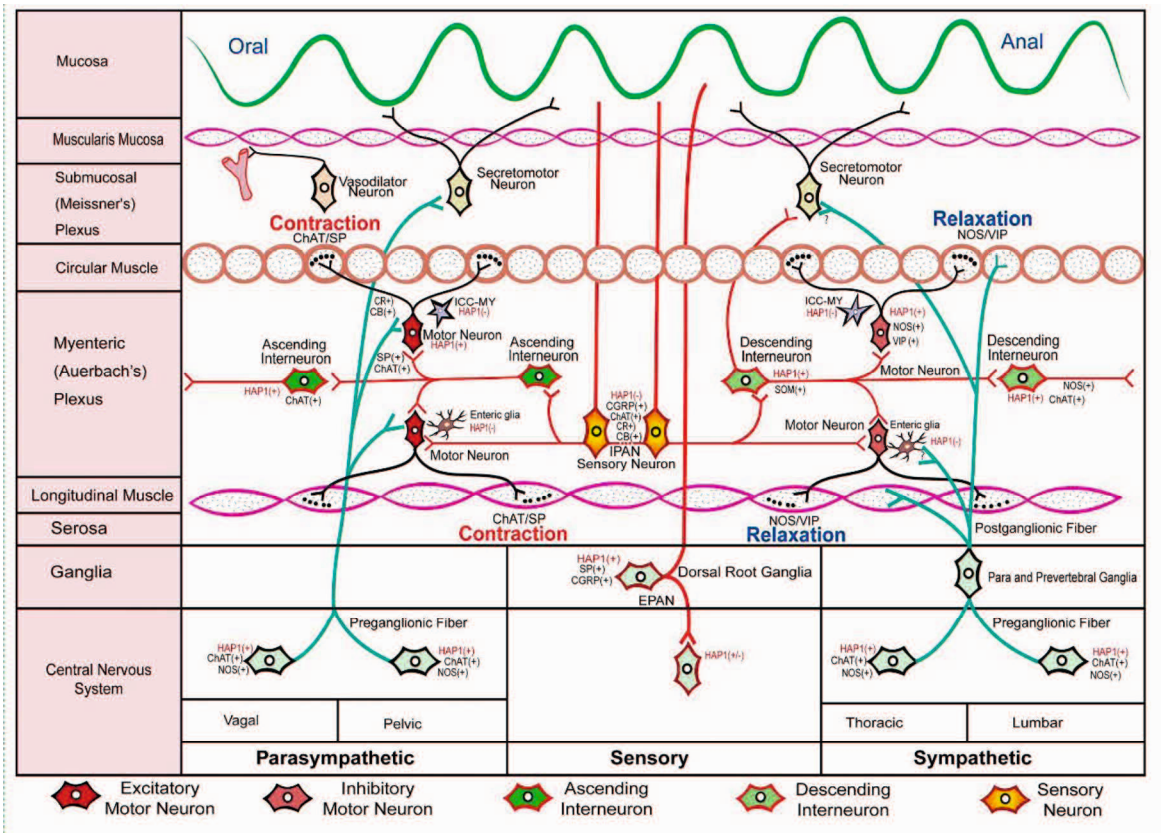

Fig. 14 Schematic illustration of the presence of HAP1-immunoreactivity in the different types of enteric neurons, neuronal circuitry and interconnection with central nervous system. This illustration is prepared on the basis of the results our current study and some other previous studies (Islam et al. 2017, 2020a; Fung et al. 2020; Furness 2012). Neuronal network of the gastrointestinal tract is innervated by intrinsic network of interconnected intrinsic sensory neuron, interneurons and motor neurons and by the axon of extrinsic sympathetic and parasympathetic autonomic neurons. Expression of different neurotransmitter is showed by positive and negative sign. In myenteric plexus, HAP1-immunoreactivity is present in the motor neurons and interneurons but not in the sensory neurons. Enteric glia and interstitial cell of Cajal (ICC-MY) in the myenteric plexus are also negative for HAP1-immunoreactivity. In addition to ENS, HAP1-immunoreactivity is also present in the spinal preganglionic autonomic neurons of sympathetic and parasympathetic regions of the central nervous system as well as dorsal root ganglia (Islam et al. 2017, 2020a). Elucidation the of neurochemical phenotypes of HAP1 in submucosal plexus is an active area of our ongoing research. ChAT, choline acetyltransferase; NO, Nitric oxide synthases; VIP, Vasoactive intestinal peptide; CR, Calretinin; CB, Calbindin; SP, Substance P; SOM, Somatostatin; CGRP, Calcitonin gene-related peptide.

Table 1 Primary antibodies used in the present study

Antibody	Immunogen	Code	Host /clonality	Source	Dilution	References
HAP1 (R19)	Rat HAP1 C-terminus	Cat# sc-8770, RRID:AB_647322	Goat polyclonal	Santa Cruz Biotechnology	1: 20,000	Islam et al., 2012
NeuN	Synthetic peptide within Human NeuN aa 1-100 (Cysteine residue)	Cat# ab177487, RRID:AB_2532109	Rabbit Monoclonal	Abcam	1:1,000	Islam et al., 2020a
HuD+HuC	Recombinant fragment within Mouse HuC aa1 to the C-terminus	Cat# ab184267, RRID:AB_2864321	Rabbit Monoclonal	Abcam	1:5,000	Lai et al., 2020
Calretinin	Recombinant human calretinin a 6-his tag at the N-terminal	Cat# CR7697, RRID:AB_2619710	Rabbit polyclonal	Swant	1:2000	Qu et al., 2008
Calbindin	Recombinant rat calbindin D-28K (CB)	Cat# CB-38a, RRID:AB_2721225	Rabbit polyclonal	Swant	1:25000	Qu et al., 2008
nNOS	C-terminal synthetic peptide sequence corresponding to amino acids (1419-1433) of human nNos coupled to KLH	Cat# 24287, RRID:AB_572256	Rabbit polyclonal	Immunostar	1:2,000	Islam et al., 2020a
VIP	Porcine VIP coupled to bovine thyroglobulin with carbodiimide linker	Cat# 20077, RRID: AB_572270	Rabbit polyclonal	Immunostar	1:5,000	Uyttebroek et al., 2013
ChAT	Synthetic peptide within Pig Choline Acetyltransferase aa 150-250.	Cat# ab178850, RRID:AB_2721842	Rabbit Monoclonal	Abcam	1:2,000	Riddle et al., 2018
SP	Synthetic Subtance P coupled to KLH with carbodiimide	Cat# 20064 RRID:AB_572266	Rabbit Polyclonal	Immunostar	1:1000	Islam et al., 2020a
Somatostatin	Human somatostatin	Cat# V1169, RRID: AB_2313730	Mouse monoclonal	Biomeda, Foster City, CA, USA	1:1,000	Yanai et al., 2020
TH	Denatured tyrosine hydroxylase from rat pheochromocytoma (denatured by sodium dodecyl sulphate)	Cat# AB152, RRID:AB_390204	Rabbit polyclonal	Millipore	1:1000	Islam et al., 2020a
CGRP	Synthetic CGRP (rat) conjugated to KLH	Cat# C8198, RRID: AB 259091	Rabbit polyclonal	Sigma–Aldrich, St. Louis, MO	1:1000	Islam et al., 2020a
GFAP	Purified human brain GFAP	Cat# G9269, RRID: AB_477035	Rabbit polyclonal	Sigma–Aldrich, St. Louis, MO	1: 1000	Islam et al., 2012
Iba-1	Synthetic peptide corresponding to the C-terminus of Iba1	Cat# 019-19741, RRID:AB_839504	Rabbit polyclonal	Wako, Osaka, Japan	1: 1000	Islam et al., 2017
C–Kit	Synthetic peptide for the C-terminal of human c-Kit	Cat# 18101, RRID:AB_494681	Rabbit Monoclonal	Immuno-Biological Laboratories Co. Ltd.	1:1,000	Hirota et al., 1998
α-tubulin	Microtubule derived from chicken embryonic brain	Cat# T6199, RRID:AB_477583	Mouse monoclonal	Sigma-Aldrich, St. Louis, MO	1:20,000	Wroblewski et al., 2018

HAP1, Huntingtin-associated protein 1; NeuN, Neuronal nuclei; nNOS, Neuronal nitric oxide synthase; VIP, Vasoactive intestinal peptide; ChAT, Choline acetyl transferase; SP, Substance P; TH, Tyrosine hydroxylase; CGRP, Calcitonin gene related peptide; GFAP, Glial fibrilliary acidic protein; Iba1, anti-ionized calcium-binding adapter molecule 1

Table 2 Co-expression ratios of HAP1 with different neuronal marker in the myenteric plexus of the gastrointestinal segments of mouse

Relationship of HAP1 with different marker (ratio%)			Stomach	Duodenum	Colon
Type	Markers	Interrelations			
Calcium binding protein	Calretinin	HAP1/Calretinin	100.0±0.0	69.5±2.1	67.75±1.8
		Calretinin/HAP1	26.5±1.2	47.75±1.3	43.75±1.6
	Calbindin	HAP1/Calbindin	100.00±0.0	45.5±2.3	42.25±1.6
		Calbindin/HAP1	24.00±1.7	10.75±0.8	12.50±1.5
Inhibitory motor neuron	NOS	HAP1/NOS	98.75±0.6	96.25±.47	97.25±.25
		NOS/HAP1	28.00±2.0	25.00±1.7	26.50±1.3
	VIP	HAP1/VIP	100.00±0.0	100.00±0.0	100.00±0.0
		VIP/HAP1	25.50±1.3	22.25±1.4	24.75±0.8
Excitatory Motor neuron	ChAT	HAP1/ChAT	100.00±0.0	71.75±1.2	68.5±1.7
		ChAT/HAP1	69.25±1.5	60.25±1.3	62.75±1.9
	SP	HAP1/SP	100.00±0.0	100.00±0.0	100.00±0.0
		SP/HAP1	28.50±1.6	28.75±1.4	26.25±1.3
Interneuron	Somatostatin	HAP1/SOM	100.00±0.0	100.00±0.0	100.00±0.0
		SOM/HAP1	1.75±0.4	2.5±0.6	3.75±0.2
Sympathetic neuron	TH	HAP1/TH	100.00±0.0	100.00±0.0	100.00±0.0
		TH/HAP1	0.25±0.02	0.17±0.04	0.27±0.04
Sensory/ IPAN	CGRP	HAP1/CGRP	0	6.25±1.0	8.75±0.6
		CGRP/HAP1	0	2.25±0.6	2.5±0.6

ChAT, Choline acetyl transferase; NOS, Nitric oxide synthase; SP, Substance P; TH, Tyrosine hydroxylase; CGRP, Calcitonin gene-related peptide; IPAN, Intrinsic primary afferent neuron; Values represent the mean ± SEM (n = 4)

Chapter V

Detailed of research objectives II

Neurochemical phenotypes of huntingtin-associated protein 1 in reference to secretomotor and vasodilator neurons in the Meissner's plexuses of rodent small intestine

5.1 Abstract

Huntingtin-associated protein 1(HAP1) is an immunohistochemical marker of stigmoid body (STB). Brain/spinal cord regions with lack of STB/HAP1 immunoreactivity are always neurodegenerative targets, whereas STB/HAP1-abundant regions tend to be spared from degeneration. In addition to central nervous system, we have recently reported that HAP1 is abundantly expressed in the excitatory/inhibitory motor neurons and interneurons in myenteric plexuses of enteric nervous system (ENS). However, HAP1 expression and its neurochemical phenotypes in Meissner's plexuses of ENS are still unknown. In this study, we aimed to clarify the expression and neurochemical characterization of HAP1 in the Meissner's plexuses of small intestine in adult mice and rats. HAP1 was highly expressed in the Meissner's plexuses of rats and mice. The percentage of HAP1-immunoreactive submucosal neurons was not significantly varied between the intestinal segments of rats and mice. Double immunofluorescence results revealed that almost all the cholinergic secretomotor neurons containing ChAT/ CGRP/ somatostatin/ calretinin, non-cholinergic secretomotor neurons containing VIP/TH/calretinin, and vasodilator neurons containing VIP/calretinin expressed HAP1. Our current study is the first to clarify that STB/HAP1 is expressed in all neuronal subgroups of Meissner's plexuses, which may reflect the involvement of HAP1 in

modulation of the secretomotor and vasodilator functions of submucosal neurons in ENS.

5.2 Introduction

Huntingtin-associated protein 1 (HAP1) was first reported as an interacting partner of huntingtin, the gene product responsible for Huntington's disease (Li et al. 1995). HAP1 is often localized to stigmoid bodies (STBs), dot-like structures found in the cytoplasm of neurons in the central and peripheral nervous systems (Gutekunst et al. 1998; Takeshita et al. 2006; Fujinaga et al. 2009; Islam et al. 2012, 2017, 2020a; Wroblewski et al. 2018). STBs were first identified in our previous studies using a polyclonal antibody raised against human placental antigen X-P2 with aromatase activity in the rat brain (Shinoda et al. 1992, 1993). HAP1 has been considered as an immunohistochemical marker of STB (Fujinaga et al. 2007, 2009; Islam et al. 2012, 2017, 2020a).

Previous *in vitro* studies showed that STB/HAP1 has protective effects against cell death in several polyglutamine neurodegenerative diseases, including Huntington's disease (Li et al. 1995), spinal and bulbar muscular atrophy (Takeshita et al. 2006), spinocerebellar ataxia type 17 (Prigge and Schmidt, 2007), Joubert syndrome (Sheng et al. 2008), and Machado-Joseph disease (Takeshita et al. 2011). In addition, the expression level of STB/HAP1 in the normal rodent brain/spinal cord varies in different areas with the highest level in the preoptic regions, hypothalamus or amygdala and low level in the cerebral cortex, thalamus, striatum, cerebellum and motoneuons of spinal cord (Li et al. 1996; Page et al. 1998; Fujinaga et al. 2004). These brain areas with low STB/HAP1 expression appear to be targets of several of neurodegenerative diseases

(Islam et al. 2017). These findings lead to the idea that STB/HAP1 is protective against neurodegenerative apoptosis/cell death (Fujinaga et al. 2004; Islam et al. 2017; Wroblewski et al. 2018). Considering normal physiological functions, the growing evidences suggest that STB/HAP1 can modulate feeding activity, postnatal growth and stress regulation (Chan et al. 2002; Li et al. 2003; Sheng et al. 2006; Xiang et al. 2014; Lin et al. 2010; Niu et al. 2011; Chen et al. 2020).

In addition to the central nervous system, STB/HAP1 is also expressed in the peripheral nervous system, including dorsal root ganglion (Islam et al. 2020) and Auerbach's plexus or Meissner's plexus in the enteric nervous system (Tarif et al. 2021). Auerbach's (myenteric) plexuses are located between the intestinal muscular layers, whereas Meissner's (submucosal) plexuses are present in the submucosal layer (Furness, 2012). We have recently clarified that HAP1 is present in excitatory motor neurons, inhibitory motor neurons, and interneurons in the myenteric plexus throughout the gastrointestinal tract (Tarif et al. 2021). Although the expression of HAP1 in the Meissner's plexus of enteric nervous system was cursorily reported in our previous study (Tarif et al. 2021), the neurochemical phenotypes of HAP1 have never been clarified there. Neurons of Meissner's plexus have been classified as cholinergic secretomotor, non-cholinergic secretomotor, and vasodilator groups (Mongardi Fantaguzzi et al. 2009). Cholinergic secretomotor neurons express ChAT, CGRP, somatostatin, and calretinin. Non-cholinergic secretomotor neurons express VIP, NOS, TH, and calretinin. Vasodilator neurons express VIP, and calretinin. Calretinin is expressed in all three subtypes of submucosal neurons, whereas, VIP is expressed in both non-cholinergic secretomotor and vasodilator neurons (Lomax and Furness, 2000; Harrington et al. 2005; Mongardi Fantaguzzi et al. 2009; Girotti et al. 2013). In addition,

a noteworthy proportion of calbindin-ir neurons are also found in submucosal plexus (Zetzmann et al. 2018). Although the functions of calbindin-ir neurons have not been clearly known in Meissner's plexus, the overwhelming majority of submucosal calbindin-ir neurons co-expressed with calretinin/VIP/somatostatin-ir neurons (Masliukov et al. 2018; Zetzmann et al. 2018).

Neurons of Meissner's plexus are involved in regulation of intestinal water and electrolyte secretion and local blood flow (vasodilation) in the gut (Furness 2006, Fung and Vanden Berghe, 2020). On the other hand, the submucosal neurons of enteric nervous system are probably most vulnerable to degeneration in neurodegenerative disease (Chalazonitis and Rao, 2018). It is possible that HAP1 might modulate certain functions of enteric nervous system or could protect submucosal neurons from neurodegenerative stress. In this context, it becomes important to examine the detailed distribution of HAP1 and its neurochemical characterization in reference to secretomotor and vasodilator neurons in the Meissner's plexuses. In this study, we aimed to examine the detailed neuroanatomical distribution of HAP1 and to elucidate its neurochemical phenotypes in the submucosal plexus in reference to the above-mentioned neuronal markers in the small intestine of adult rats and mice.

5.3 Materials and methods

5.3.1 Animals

8-10 weeks of aged mice (C57BL/6J) of either sex, were maintained in a cage under standard conditions in an artificially lit room with a 12-h light/dark cycle at 21°C. All mice were supplied *ad libitum* with water and standard pellet diet. The animals were obtained from Japan SLC Inc., Shizuoka, Japan. The basic considerations for the

handling of laboratory animals were followed by the guidelines for Animal Research of Japanese Government's Law (No. 105) and Notification (No. 6). The Committee on the Ethics of Animal Experimentation at Yamaguchi University School of Medicine formulated voluntary in-house regulations for proper scientific conduct of animal experiments and welfare based on these Guidelines.

5.3.2 Tissue section preparation

Animal were anesthetized with sodium pentobarbital (80mg/kg). The mice were perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4) and post-fixed for overnight at 4°C in same fixative. The following day, the experimental segments (duodenum, jejunum, ileum) were cut into 1-1.5cm full thickness rings. It was washed properly with dimethyl sulphoxide (DMSO, 3×10 min) and placed in PBS containing 0.1% sodium azide and 30% sucrose solution for cryoprotection and stored at 4°C for three days. Optimal cutting temperature/OCT medium (Sakura Finetek USA, Inc., Torrance, CA, USA) was used to embed the tissue and then quick-freezed using powdered dry ice. Finally, tissue sections (30μm) were prepared frontally by using cryostat.

5.3.3 Whole mount preparation

For whole mount preparations, segments (duodenum, jejunum, ileum) were cut along the mesenteric border, opened, flatted in the Sylgard-lined Petri dish and washed with phosphate buffer saline (PBS). The tissue was dissected to remove the mucosa and external muscle and producing the submucosal layer consisting of the submucosal plexus under a dissection microscope. Tissue sections were cleaned of fixative in 100% dimethyl sulphoxide (3×10 min washes) and then in PBS (3×10 min washes).

5.3.4 Single immunoperoxidase immunohistochemistry

Immunoperoxidase immunohistochemistry was performed as described by Islam et al. 2012, 2017. To prevent nonspecific antibody binding and to reduce endogenous peroxidase activity, samples were incubated with 10% normal donkey serum (NDS) and 0.3% Triton X-100 in PBS for 1h at 4°C. Then, a mixture of 1.5% hydrogen peroxide plus 50% methanol was used to bleach the samples for 30 min at 4°C. Thereafter, samples were incubated with primary antibodies (Table 1) diluted in PBs containing 0.3% Triton X-100 and 1% NDS (PBST-NDS) at 20°C for 4 days. Primary antibodies used in the present research available commercially. The antibodies used were as follows: goat anti-HAP1 (R19; 1:20,000), rabbit anti-neuronal nuclei (NeuN; 1: 1,000), rabbit anti-HuD+HuC (1:5,000), rabbit anti-VIP (1:5,000), rabbit anti-NOS (1:2,000), rabbit anti-ChAT (1:2,000), rabbit anti-CGRP (1:1,000) antibody, rabbit anti-calretinin (1:2,000), rabbit anti-calbindin (1:25,000), rabbit anti-somatostatin (1:1,000), and rabbit anti-TH (1:1,000). Then, samples were incubated with appropriate biotinylated secondary antibodies labelled with donkey anti-goat IgG (AP180B, Millipore; 1:1,000) and donkey anti-rabbit IgG (Dako, Denmark; 1:1,000) diluted in PBS containing 1% NDS at 4°C for overnight followed by incubation with horseradish peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark; 1:1,000) diluted in PBS for 2h at 20°C. The location of antigen/antibody complex was detected using 3,3′-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and nickel ammonium sulfate (Sigma– Aldrich, Tokyo, Japan) in 0.05 M Tris–HCl buffer containing 0.0008% hydrogen peroxide for violet to black color reaction. Finally, sections or whole mount preparations were air dried, dehydrated using an ethanol gradient and mounted on glass slides.

5.3.5 Double immunofluorescence immunohistochemistry

The samples were prepared for double immunofluorescence immunohistochemistry as described by Jahan et al. 2015. Samples were incubated with antiserum buffer solution (PBS containing 10% normal donkey serum (NDS), 0.3% Triton X-100) for 2h at 4°C to prevent non-specific bindings, followed by incubation with goat anti-HAP1 primary antibody (R19, 1:20,000), diluted in 1% PBST-NDS as described. Antibodies from different species were incubated simultaneously (Table 1). To increase the immunoreactivity of neuronal cell bodies in case of using VIP, TH, and CGRP antibodies, colchicine with 0.9% NaCl saline solution was injected intraperitonially for both single and double label staining (Tarif et al. 2021). After washing (3×10 min) in PBS, samples were incubated with Alexa Fluor 594 donkey anti-goat IgG (Invitrogen, USA) against HAP1 antibody and Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen, USA) against Hu, NeuN, ChAT, CGRP, calretinin, calbindin, Somatostatin, TH, NOS, VIP antibodies for 2-3hr at 20°C followed by three 10-min washes in PB. Then, all samples were mounted on glass slides by using mounting medium (Fluoromount/Plus, K048, Diagonostic Biosystems, USA).

5.3.6 Image processing

Single Immunoperoxidase immunohistochemistry photographs were recorded using an Eclipse E80i photomicroscope (Nikon, Tokyo, Japan) and a color digital camera (Lumenera Corporation, Ottawa, Canada). Double immunofluorescence photomicrographs were analyzed by confocal microscope/LSM510 (Carl Zeiss, Jena, Germany) on a Zeiss Pascal confocal laser scanning system. Photos were processed by changing the filter to adjust the contrast and brightness only. Adobe Photoshop Elements 2020 was utilized to organize the photographs.

5.3.7 Cell counting

Colocalization analysis of HAP1 and other marker were conducted through double labeling of the whole mount samples. The cells were detected on the presence of the antigen-marking fluorophore and clearly visible cells with nucleus were counted. By changing the filter, submucosal neurons were detected by a second antigen-marking fluorophore. Random images were chosen and immunoreactive neuronal profile were counted in a total of 10 fields (0.8mm^2 /microscopic field for immunoperoxidase staining) and 0.25mm^2/microscopic field for immunofluorescence staining) in each mouse (Tarif et al. 2021). A total of 5 mice were used for each quantification. Data were expressed as mean ± standard error of the mean (SEM; n =4).

5.3.8 Statistical analysis

One-Way ANOVA (analysis of variance) was done to reveal any difference among different parts of small intestine in mice and rats. Also, parts-specific differences between mouse and rats were revealed by Student's t-test. P values of <0.05 were considered statistically significant. A software package SPSS version 22 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

5.4 Results

5.4.1 Expression pattern of HAP1-ir cells in the submucosal ganglion

To examine the detailed distribution of HAP1in the submucosal plexus of small intestine, single immunoperoxidase immunohistochemistry was performed using whole-mount preparations of duodenum, jejunum and ileum of mice and rats. Strongly stained HAP1-ir cells were present in the submucosal ganglion throughout the different segments of small intestine of mice (Fig. 15 A-C) and rats (Fig. 15 D-F). Dot like stigmoid bodies were observed in the cytoplasm of most of the HAP1-ir cells, however

there were some HAP1-ir cells were also present without containing clearly visible STBs in their cytoplasm in the submucosal ganglion of small intestine of mice (Fig. 15 A-C) and rats (Fig. 15 D-F). The distribution pattern of HAP1-ir cells was generally similar in different segments (duodenum, jejunum and ileum) of both rodents (Fig. 15 A-F). The frequencies of occurrences of HAP1-ir cells in submucosal plexuses were not significantly varied among the intestinal segments of same species of rodents ($P>0.05$; one-way ANOVA), however the number of HAP1-ir cells were significantly higher in the submucosal plexuses in intestinal segments of rats than that of mice ($P<0.005$; Student's t-test) (Fig.15 G).

Here question arises, this significant difference in the number HAP1-ir cell is due to species difference in HAP1 expression or due to the species difference in the total number of neurons in the submucosal plexus in small intestine. To answer this question, we examined the expression of pan neuronal marker Hu in duodenum, jejunum and ileum of mice and rats (Fig. 16A-F). Our cell counting results revealed that the number of Hu-ir cells were significantly higher in the submucosal plexuses in intestinal segments of rats than that of mice ($P<0.005$; Student's t-test) (Fig. 16 G). This indicated that the significant difference of HAP1-ir cells in the submucosal plexuses in intestinal segments of rats than that of mice was due to the difference in total number of neurons in submucosal plexuses.

Next, profile counting of HAP1-ir cells was performed by comparing the total number of Hu with the total number of HAP1-positive neurons (Fig. 16 A-R). Our current result clearly revealed that the percentage of HAP1-ir cells in the submucosal plexus of different intestinal segments ranged between 89–91% in mice (Fig. 17 A-I) and 87-91% in rats (Fig. 17 J-R). However, significant difference in the percentage of HAP1-ir submucosal neurons was not observed among the intestinal segments of same species

of rodents ($P>0.05$; one-way ANOVA). Similarly, the percentage of HAP1-ir submucosal neurons was also not significantly varied between the intestinal segments of rats and mice ($P<0.005$; Student's t-test) (Fig. 18).

To reveal the relationships of HAP1 with neuronal marker (NeuN) we performed the double-label immunohistochemistry of HAP1 with NeuN in jejunum. Our result showed that all the NeuN are co expressed with HAP1 meaning that HAP1-ir cells demonstrated attributes of neurons (Fig. 19). Next, we checked the expression of different functional markers such as Hu (A), VIP (B), NOS (C), ChAT (D), calretinin (E), calbindin (F), Somatostatin (G), CGRP (H) and TH (I) in the submucosal plexus of mouse jejunum by using single immunoperoxidase staining (Fig. 20).

5.4.2 Relationships of HAP1 with the markers of cholinergic secretomotor neurons

To reveal the relationships of HAP1 with the markers of cholinergic secretomotor neurons in the submucosal plexus, first we performed the double-label immunohistochemistry of HAP1 with ChAT in jejunum. Our cell counting data indicated that 100% of ChAT neurons had HAP1 immunoreactivity in jejunum of both mouse and rat. Conversely, of HAP1-immunoreactive cell bodies, 44% and 46% were ChAT immunoreactive in jejunum of both mouse and rat respectively (Fig. 21 A-F, Table 3).

Next, co-expression ratio of HAP1 with CGRP was determined. Our current double-immunofluorescence results revealed 100% of CGRP neurons had HAP1 immunoreactivity in jejunum of both mouse and rat. Conversely, of HAP1-immunoreactive cell bodies, 41% and 45% were CGRP immunoreactive in jejunum of

both mouse and rat respectively (Fig. 22 A-F, Table 3). In addition, our current study also revealed that100% of somatostatin neurons had HAP1 immunoreactivity in jejunum of both mouse and rat. Conversely, of HAP1-immunoreactive cell bodies, 28% and 31% were somatostatin-immunoreactive in jejunum of both mouse and rat respectively (Fig. 22 G-L, Table 3).

5.4.3 Relationships of HAP1 with the markers of non-cholinergic secretomotor and Vasodilator neurons

To reveal the relationships of HAP1 with the markers of cholinergic secretomotor neurons in submucosal plexus, first performed the double-label immunohistochemistry of HAP1 with VIP, NOS or TH in jejunum. Our cell counting data revealed that 100% of VIP neurons had HAP1 immunoreactivity in jejunum of both mouse and rat. Conversely, of HAP1-immunoreactive cell bodies, 54% and 53% were VIP-immunoreactive in jejunum of both mouse and rat respectively (Fig. 23 A-F, Table 3). Our cell counting results also showed that 100% of NOS neurons had HAP1 immunoreactivity in jejunum of both mouse and rat. Conversely, of HAP1-immunoreactive cell bodies, 2% and 9% were NOS-immunoreactive in jejunum of both mouse and rat respectively (Fig. 23 G-L, Table 3).

Furthermore, cell counting data showed that 100% of TH neurons had HAP1 immunoreactivity in jejunum of both mouse and rat. Conversely, of HAP1-immunoreactive cell bodies, 22% was TH-immunoreactive in jejunum of mouse (Fig. 24 A-C, Table 3). In case of rat, there is no cell counting data as we did not able to detect the cell body of TH (Fig. 24 D-F).

Finally, we performed double-label immunohistochemistry of HAP1 with calretinin and calbindin. Calretinin and calbindin are expressed in diverse group of

submucosal neurons (Mongardi Fantaguzzi et al. 2009; Zetzmann et al., 2018). Our current results demonstrated that 100% of calretinin neurons had HAP1 immunoreactivity in jejunum of both mouse and rat. Conversely, of HAP1-immunoreactive cell bodies, 94% and 92% were calretinin-immunoreactive in jejunum of both mouse and rat respectively (Fig. 25 A-F, Table 3). Furthermore, 100% of calbindin neurons had HAP1 immunoreactivity in jejunum of both mouse and rat. Conversely, of HAP1-immunoreactive cell bodies, 34% and 42% were calbindin-immunoreactive in jejunum of both mouse and rat respectively (Fig. 25 G-L, Table 3).

5.5 Discussion

The present research is the first to examine the detailed distribution of HAP1in the submucosal plexus of intestine in adult mouse. The current study is also the first to elucidate the neurochemical phenotypes of HAP1 immunoreactivity in reference to cholinergic secretomotor, non-cholinergic secretomotor, and vasodilator neurons in the submucosal plexus. To date, the distribution of HAP1 has been extensively analyzed in the brain (Gutekunst et al., 1998; Fujinaga et al., 2004, Dragatsis et al., 2004; Sheng et al., 2008; Lin et al., 2010; Niu et al., 2011; Islam et al., 2012, 2017; Wroblewski et al., 2018; Chen et al., 202), spinal cord (Islam et al., 2017), dorsal root ganglion (Islam et al., 2020a), and in myenteric plexus of enteric nervous system (Tarif et al., 2021). Nevertheless, the detailed distribution and neurochemical characterization of HAP1 have never been examined in the Meissner's plexuses of enteric nervous system. Using immunoperoxidase and immunofluorescent immunohistochemical technique, the current study determined the distribution and neurochemical characterization of HAP1-ir cells in the submucosal plexus in mouse and rat small intestine. Our current results showed that the number of Hu-ir cells were significantly higher in the submucosal

plexuses in intestinal segments of rats than that of mice, however, the profile counting of HAP1-ir cells (comparing the total number of Hu with the total number of HAP1-positive neurons) indicted that the percentage of HAP1-ir submucosal neurons was also not significantly varied between the intestinal segments of rats and mice. Neurochemical characterization of HAP1 revealed that all the secretomotor and vasodilator neurons express HAP1 immunoreactivity (Fig. 26). The current study might reflect HAP1's involvement in regulation of the secretomotor (activate secretion) and vasodilator (reduce secretion) functions of enteric neurons.

In the submucosa of mouse small intestine, there are three classes of neurons, such as non-cholinergic secretomotor neurons, cholinergic secretomotor neurons, and vasodilator neurons (Mongardi Fantaguzzi et al. 2009). These submucosal neurons regulate the movement of water and electrolytes across the mucosal epithelium (Furness et al. 2003). The cholinergic and non-cholinergic secretomotor neurons are uniaxonal projecting short distances (often < 1mm) and display S-type electrophysiology (Mongardi Fantaguzzi et al. 2009; Wong et al. 2008; Foong et al. 2014). The secretomotor and vasodilator neurons supply to either the mucosa or vasculature separately instead of supplying to both, suggesting that common diverging inputs are required to regulate blood flow and intestinal secretion (Mongardi Fantaguzzi et al. 2009). Cholinergic secretomotor neurons express ChAT, CGRP, somatostatin, and calretinin, whereas, non-cholinergic secretomotor neurons express VIP, NOS, TH, and calretinin. However, vasodilator neurons express VIP, and calretinin (Lomax and Furness, 2000; Harrington et al., 2005; Mongardi Fantaguzzi et al. 2009; Girotti et al., 2013). Previous quantitative data indicate that VIP is present about 50%, ChAT is present about 42%, calretinin is present approximately 91%, CGRP is expressed about 31%, somatostatin is present about 30%, NOS is expressed about 29%, and TH is

present about 22% of total neurons in the submucosal plexus of mouse (Mongardi Fantaguzzi et al., 2009). Our current study revealed an intriguing result that all the neurochemical markers of submucosal neurons showed a very high coexpression ratio with HAP1-ir cells (co-expression about 100%). Taking the methodological limitations into account, our current results may indicate that most of the secretomotor and vasodilator neurons contain HAP1 immunoreactivity. However, in our current study, we found a very small population of Hu-ir neurons that did not contain HAP1-immunoreactivity, indicating that those neurons were not secretomotor and vasodilator type. These results are consistent with the previous report (Mongardi Fantaguzzi et al., 2009), where they stated that there were about 8-10% submucosal neurons which were neither ChAT or VIP-immunoreactive.

Neurons of submucosal plexus are involved in modulation of intestinal water and electrolyte secretion and local blood flow (vasodilation) in the gut (Furness, 2006, Fung and Vanden Berghe, 2020). Vasodilator and secretomotor reflexes are crucial for intestinal blood flow and secretion that can be triggered by different chemical or mechanical stimulation. (Christofi et al., 2004; Hansen and Witte, 2008). Vasodilation is occurred by the activation of muscarinic M3 receptor onto endothelial cells (Vanner and Surprenant, 1996). Whereas, mucosal muscarinic M3 receptors or VPAC1 receptors are activated to elevate intracellular Ca^{2+} and cAMP for triggering secretory responses (Xue et al., 2007). Although further detailed physiological and morphological studies need to be performed in the future to examine how HAP1 affects the activation of mucosal or endothelial M3/VPAC1 receptor expression to modulate vasodilator and secretomotor reflexes, our present results might serve as basis for the elucidation of HAP1-dependent regulation secretomotor/vasodilator functions of submucosal neurons.

On the other hand, HAP1 can sequester the pathological mutant molecules and trap the toxic aggregation in the cytoplasm to prevent apoptosis-inducing nuclear translocation in different neurodegenerative diseases (Takeshita et al., 2006, 2011). In addition, the brain/spinal cord regions with low HAP1-expresson are always target for neurodegeneration, whereas the regions with high HAP1-expression are spared from neurodegeneration (Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Islam et al. 2012, 2017). In addition to brain/spinal cord, ENS is also a neurodegenerative target for different neurodegenerative disorders (Chalazonitis and Rao, 2018). Furthermore, the ENS can act as a potential portal for pathogenesis of neurogenerative disorders (Kuwahara et al., 2020; Sharon et al., 2016). The Braak hypothesis states that pathologic α-synuclein enters into the gastrointestinal tract to the ventral midbrain via the vagus nerve, where there is loss of dopaminergic neurons in substantia nigra pars compacta (Kim et al., 2019). Our current results showed that HAP1 was highly present in submucosal plexus. In our previous study, we found that HAP1 was also abundantly expressed in myenteric neurons (Tarif et al., 2021). Taken together, due to putative protective functions, abundant HAP1 expression in both the myenteric and Meissner's plexus suggests that HAP1-immunoreactivity may give out beneficial stability to enteric neurons from neurodegeneration. It is intriguing to consider that the individual with decreased HAP1-expression or mutated HAP1 or in the ENS might be more vulnerable to neurodegenerative diseases. It has been reported that HAP1 is significantly reduced in different cancer tissues, such as breast cancer (Zhu et al., 2013), pancreatic cancer (Li et al., 2019), suggesting that HAP1 can serve as a potential diagnostic marker for certain cancer. Downregulated or mutated HAP1-expression in ENS could be a potential risk factor for certain neurodegenerative disorders. Changes in the composition of gut microbiota in the GIT are believed to link with the

pathogenesis of neurological disorders in ENS and CNS (Sugunya and Koo, 2020). Now a days the microbiota/gut-and-brain axis is an emerging and widely accepted concept. HAP1 might affect the colonization of microbiota in intestine and consequently affect the neurodegeneration in ENS and CNS. Therefore, future studies should include detailed *in vitro* and *in vivo* studies to clarify the effects of HAP1 on the vulnerability of enteric neurons regarding neurodegenerative disorders to explore new diagnostic/therapeutic paradigms of certain neurodegenerative diseases utilizing tissues from the gastrointestinal tract.

In conclusion, our present study is the first to examine the expression HAP1 in the Meisner’s plexus of ENS. The present study is also the first to clarify the characterization of HAP1-ir cells in refence to secretomotor and vasodilator submucosal neurons. Our current results reveal that HAP1 is expressed in all the secretomotor and vasodilator neurons. The current study might reflect the involvement of HAP1 in modulation of the secretomotor and vasodilator functions of submucosal neurons. Our current results lay a basic foundation for future studies that seek to clarify the physiological and pathological roles of HAP1 in the Meisner’s plexus of ENS.

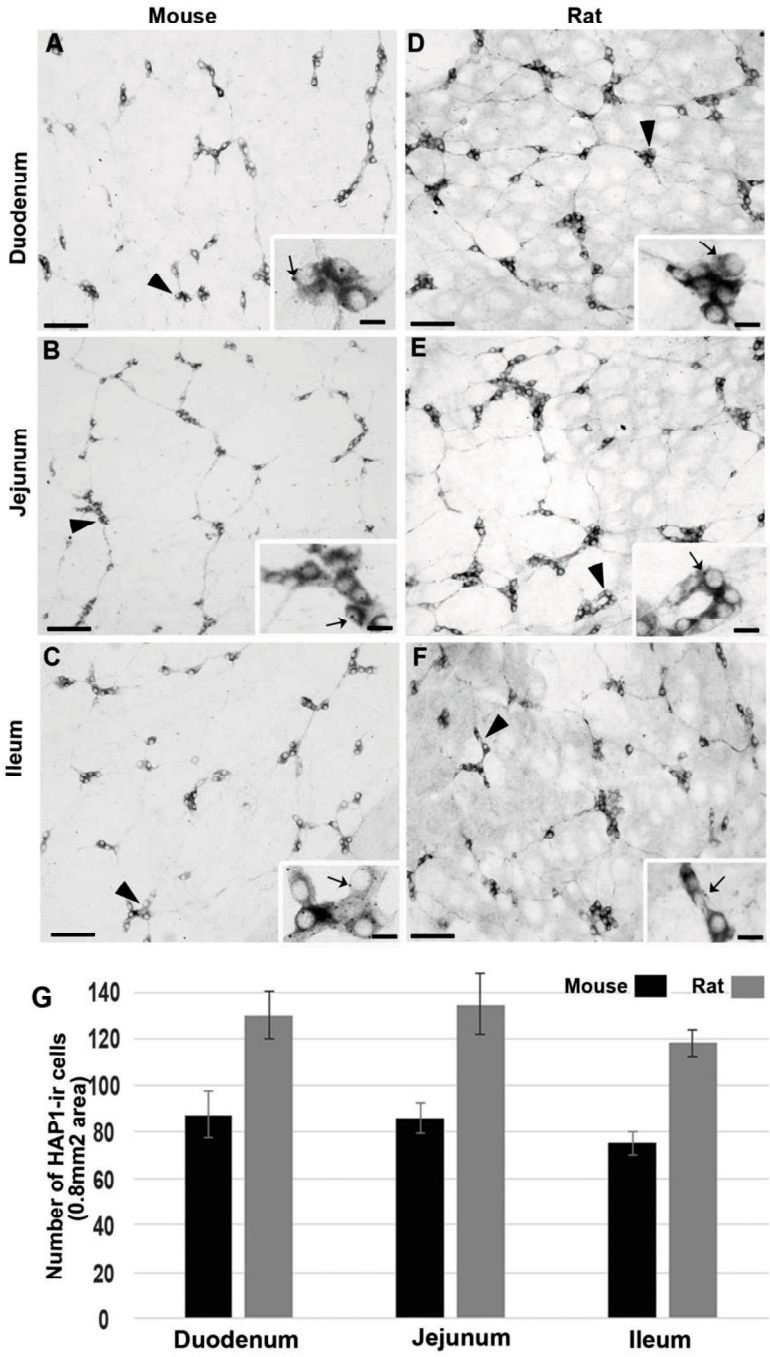

Fig. 15 Quantification of huntingtin-associated protein 1 (HAP1) in whole mount preparation in mouse and rat (Single immunoperoxidase staining). Photomicrographs showing HAP1-ir cells in the submucosal plexus in duodenum, jejunum, and ileum in mouse (A-C) and rat (D-F). Scale bar =100μm (a-f). Counting of HAP1-ir cells in the submucosal plexus of mouse and rat in duodenum, jejunum and ileum (G). Immunoreactivity of HAP1 was counted in a microscopic field of 0.8mm^2 in 10 nonoverlapping 10X fields (n=4).

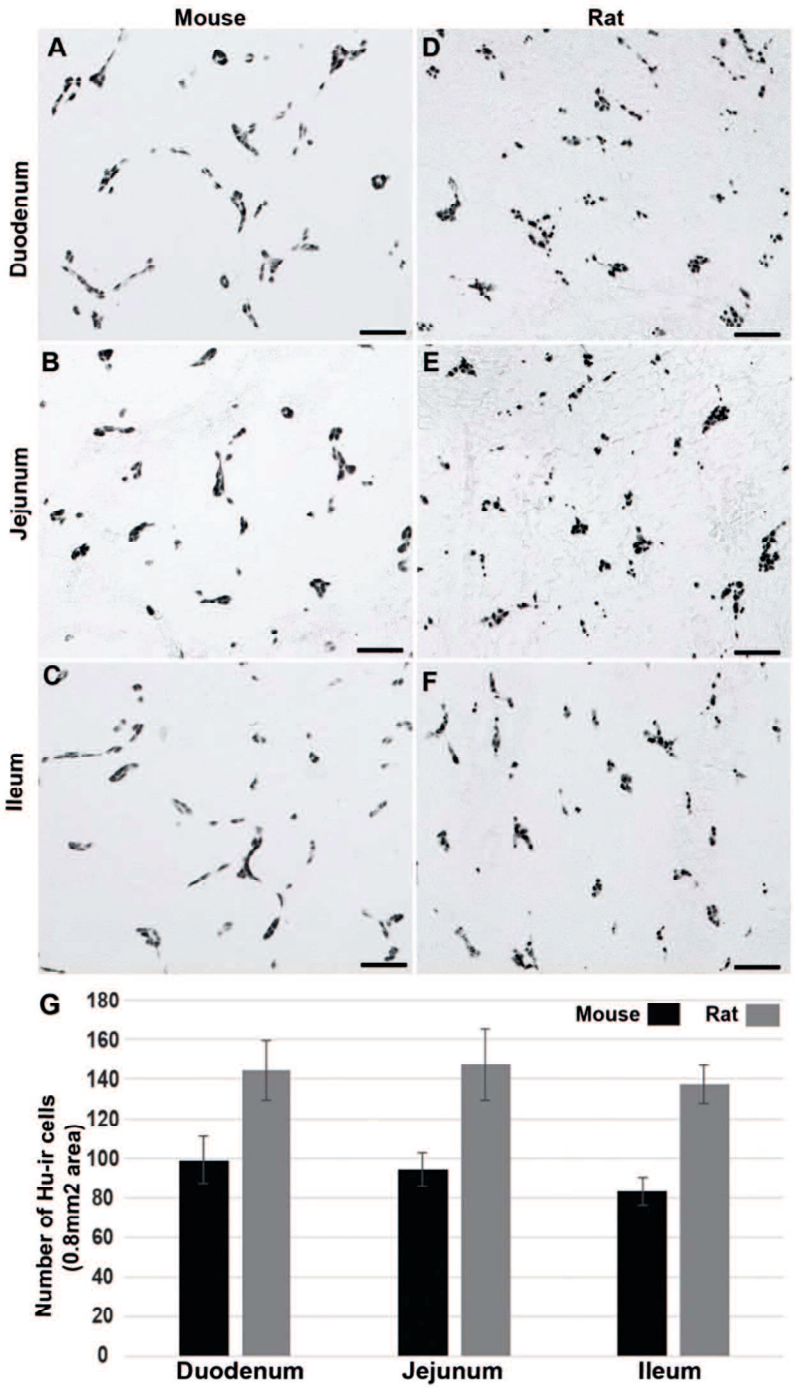

Fig. 16 Quantification of Hu in whole mount preparation in mouse and rat (Single immunoperoxidase staining). Photomicrographs showing Hu-ir cells in the submucosal plexus in duodenum, jejunum and ileum in mouse (A-C) and rat (D-F). Scale bar =100μm (A-F). Counting of Hu-ir cells in the submucosal plexus of mouse and rat in duodenum, jejunum and ileum (G). Immunoreactivity of HAP1 was counted in a microscopic field of 0.8mm^2 in 10 nonoverlapping 10X fields (n=4).

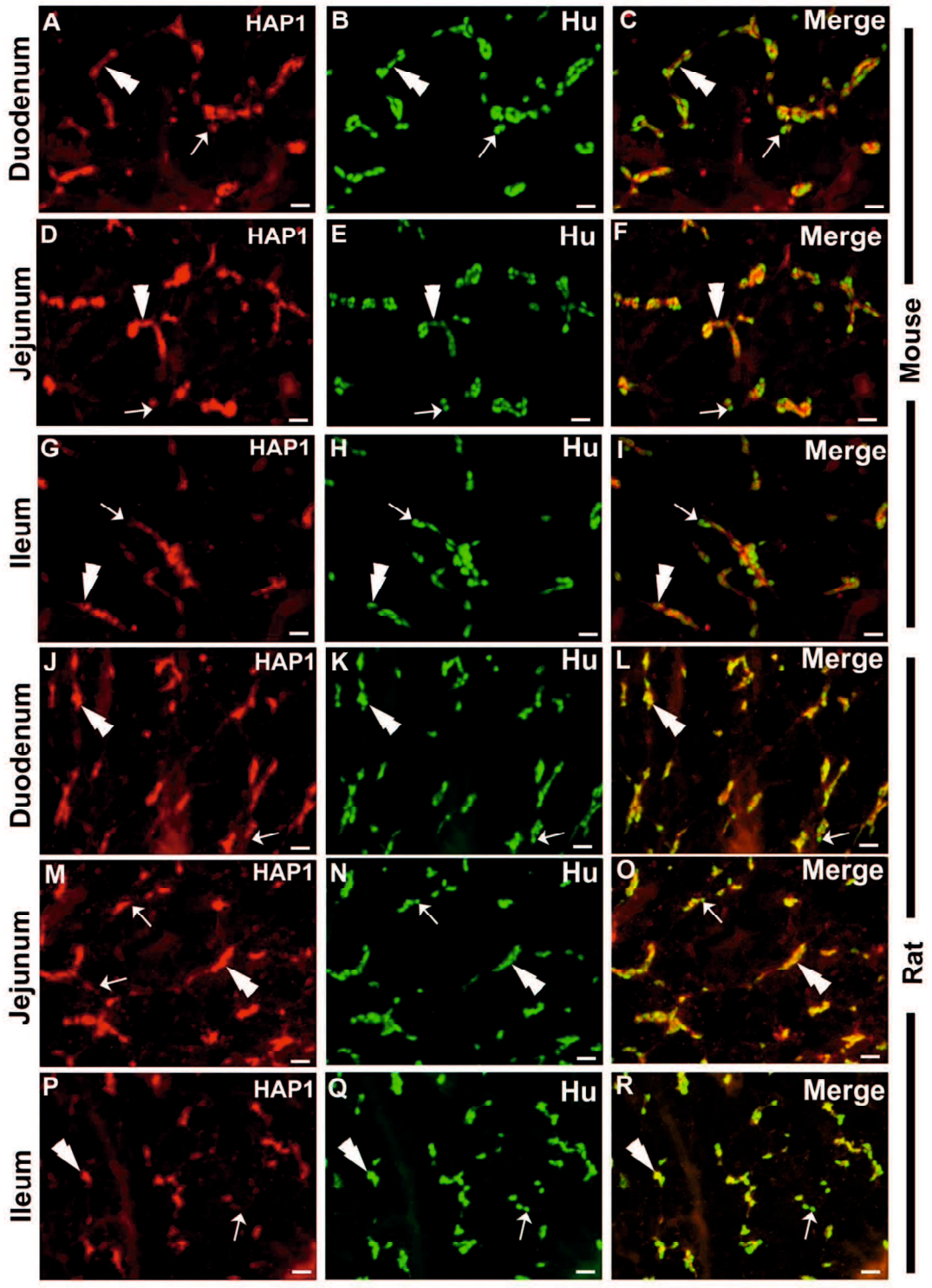

Fig. 17 Photomicrographs showing double-label immunofluorescence immunohistochemistry of HAP1 and Hu in the submucosal plexus of duodenum, jejunum and ileum in whole mount preparations of mouse and rat (A-R). Cells positive for both HAP1 and Hu is indicated by double arrowheads. Hu Single-positive cell is indicated by arrows. Scale bar =50μm (A-R).

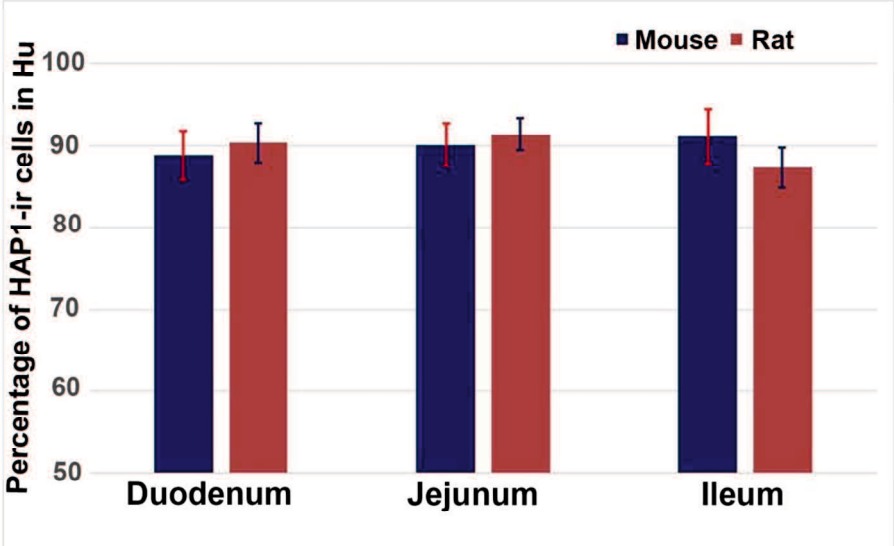

Fig. 18 The graphs showing coexpression ratios of HAP1 visualized with the pan-neuronal marker Hu-immunoreactivity (ir) in the submucosal plexus of mouse small intestine. Immunoreactivity of HAP1/ Hu was counted in a microscopic field of 0.25mm^2 in 10 nonoverlapping 20X fields. Values represent the mean ± SD (n = 4).

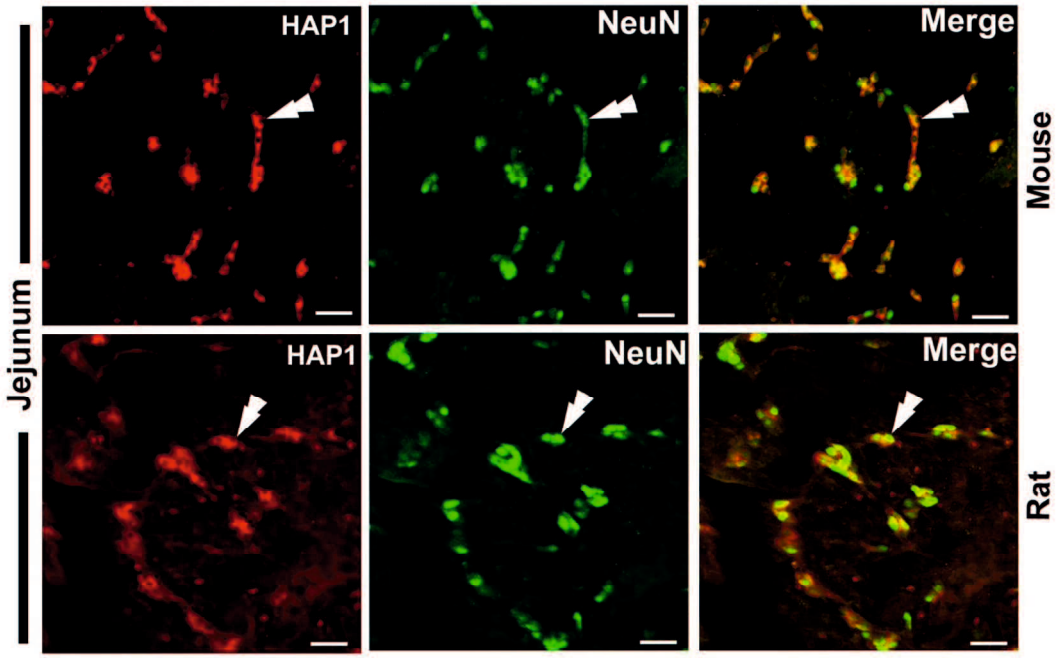

Fig. 19 Photomicrographs showing double-label immunofluorescence immunohistochemistry of HAP1 and NeuN in the submucosal plexus of mouse jejunum (A-F) in whole mount preparations. Cells positive for both HAP1 and NeuN is indicated by double arrowheads. Scale bar =50μm (A-F).

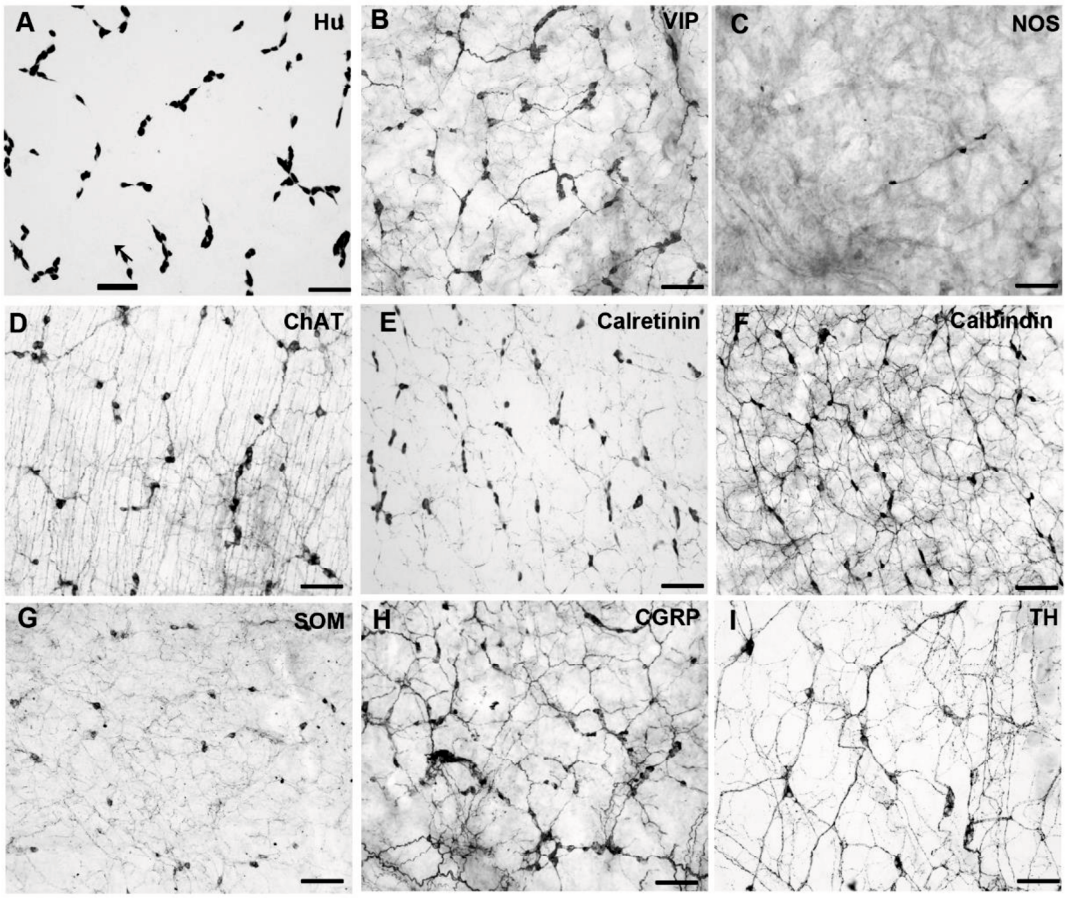

Fig. 20 Expression of different functional markers such as Hu (A), VIP (B), NOS (C), ChAT (D), calretinin (E), calbindin (F), Somatostatin (G), CGRP (H) and TH (I) are detected in the submucosal plexus of mouse jejunum. Scale bar =100μm (A-J) and 10μm in insets.

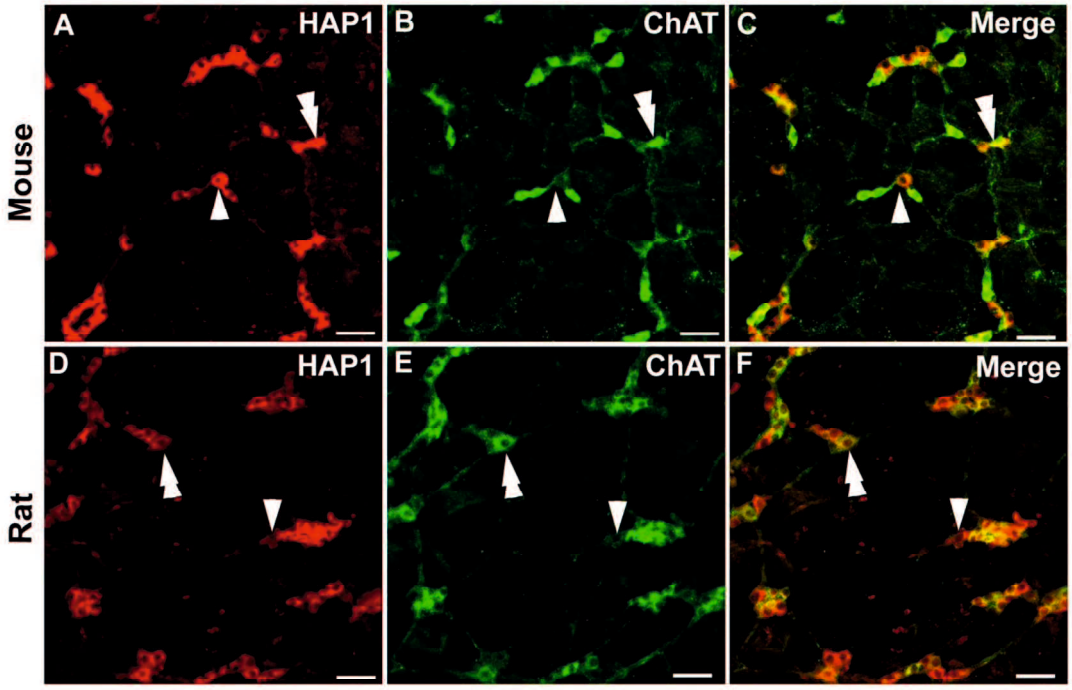

Fig. 21 Photomicrographs showing double-label immunofluorescence immunohistochemistry of HAP1 with ChAT in the submucosal plexus of jejunum in whole mount preparations of mouse (A-C) and rat (D-F). Cells positive for both HAP1 with ChAT are indicated by double arrowheads. HAP1 single positive cell is indicated by arrowhead. Scale bar =50μm (A-F).

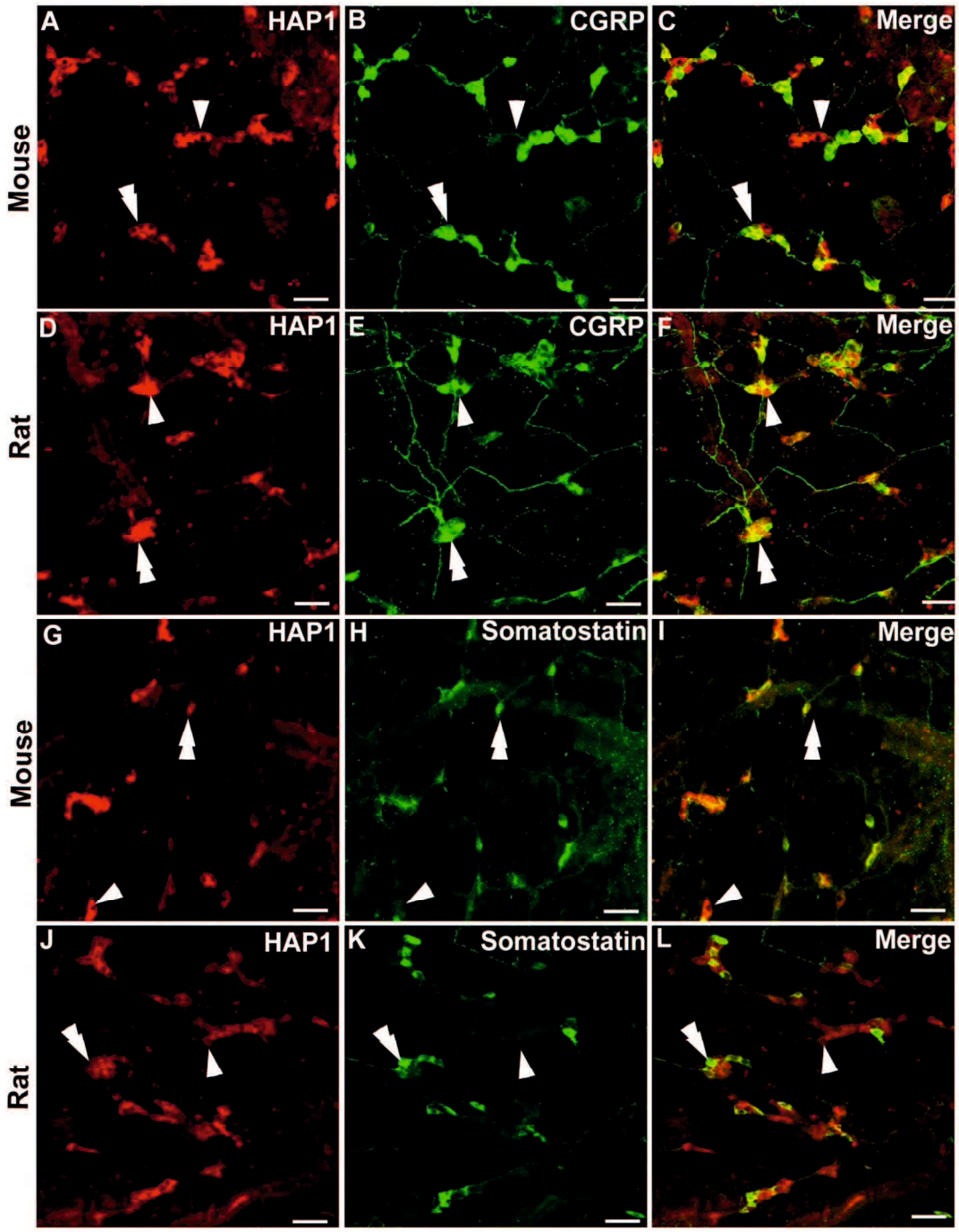

Fig. 22 Photomicrographs showing double-label immunofluorescence immunohistochemistry of HAP1 with CGRP (A-F) and HAP1 with somatostatin (G-L) in the submucosal plexus of jejunum in whole mount preparations of mouse and rat. Cells positive for both HAP1 with CGRP and HAP1with somatostatin are indicated by double arrowheads. HAP1 single positive cell is indicated by arrowhead. Scale bar =50μm (A-L).

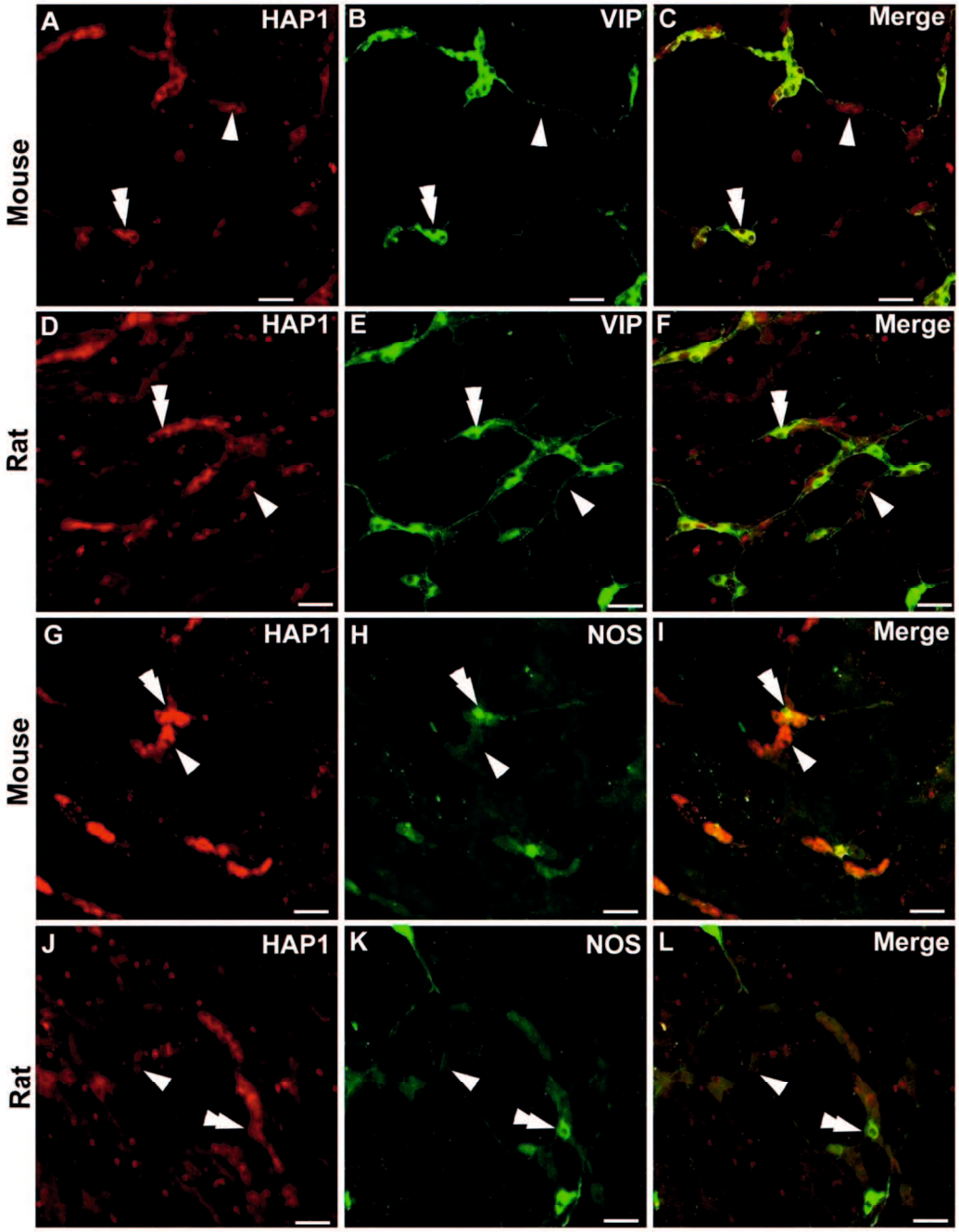

Fig. 23 Photomicrographs showing double-label immunofluorescence immunohistochemistry of HAP1 with VIP (A-F) and HAP1 with NOS (G-L) in the submucosal plexus of jejunum in whole mount preparations of mouse and rat. Cells positive for both HAP1 with VIP and HAP1 with NOS is indicated by double arrowheads. HAP1 single positive cell is indicated by arrowhead. Scale bar =50μm (A-I).

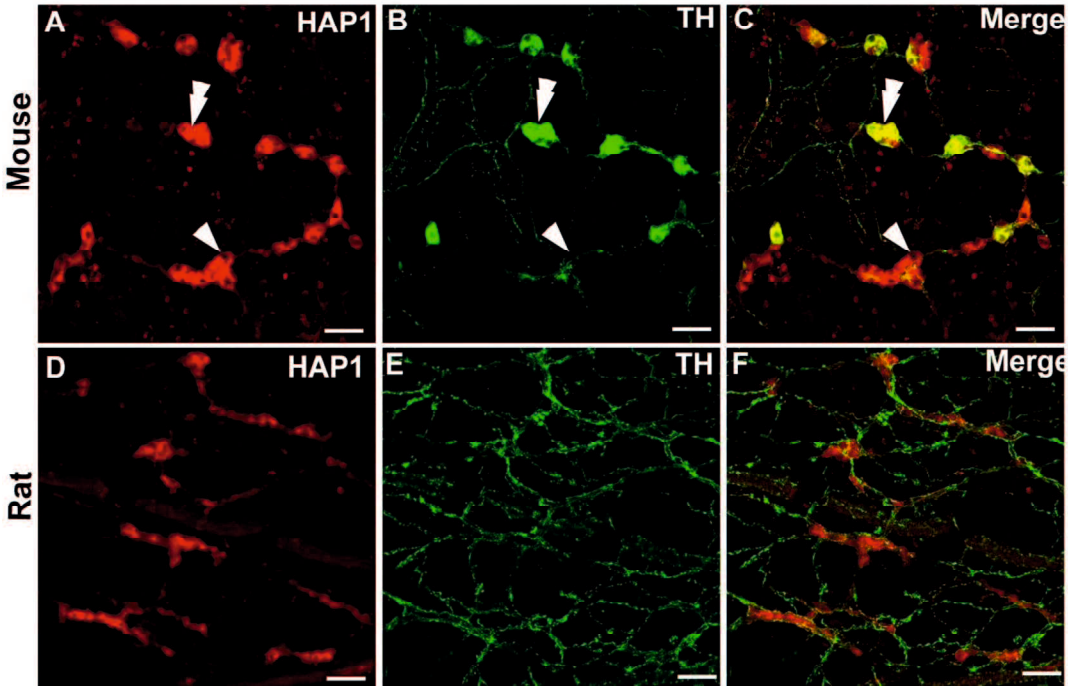

Fig. 24 Photomicrographs showing double-label immunofluorescence immunohistochemistry of HAP1 with somatostatin in the submucosal plexus of jejunum in whole mount preparations of mouse (A-C) and rat (D-F). Cells positive for HAP1with TH are indicated by double arrowheads. HAP1 single positive cell is indicated by arrowhead. Scale bar =50μm (A-F).

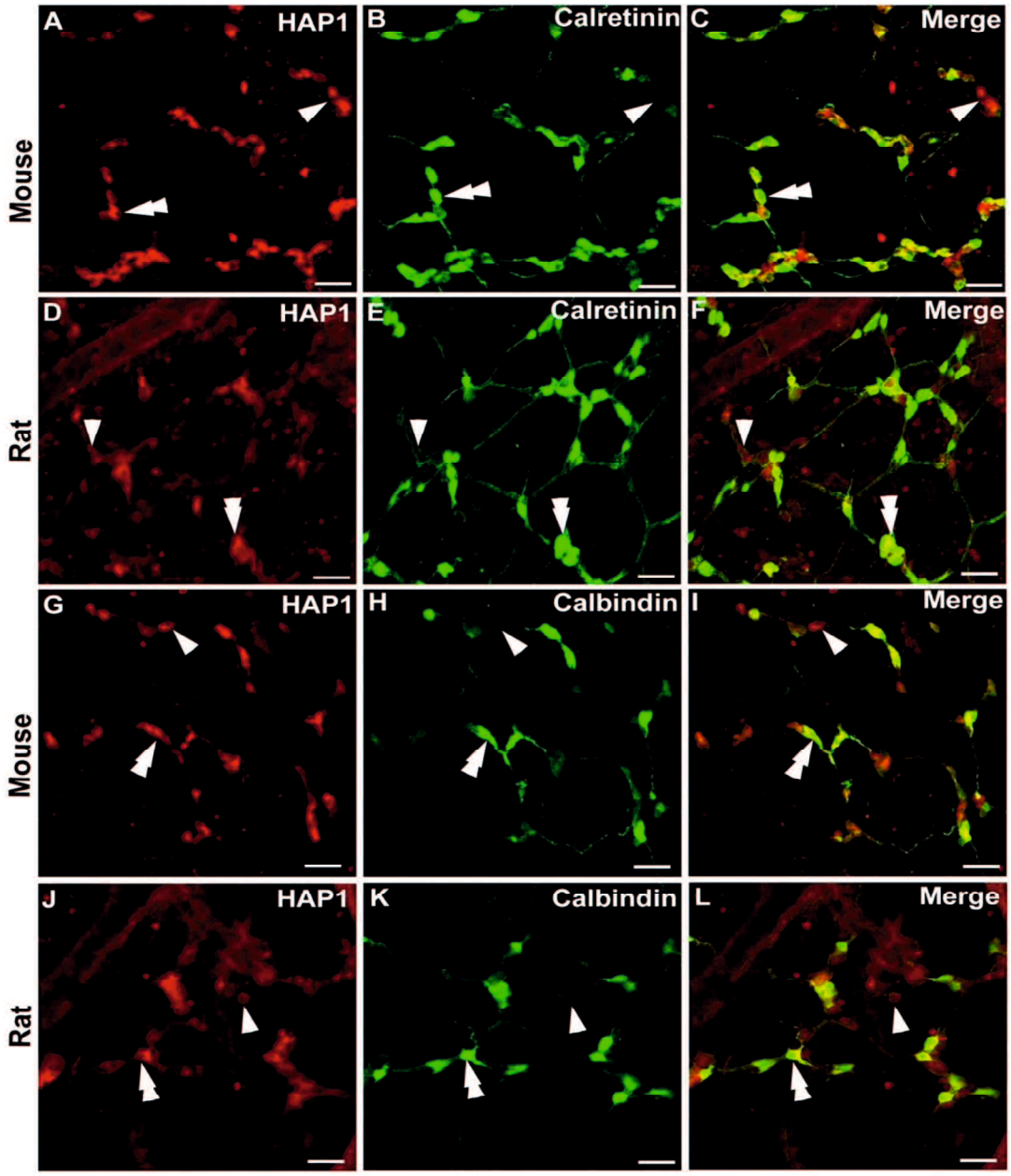

Fig. 25 Photomicrographs showing double-label immunofluorescence immunohistochemistry of HAP1 with calretinin (A-F) and HAP1 with calbindin (G-L) in the submucosal plexus of jejunum in whole mount preparations of mouse and rat. Cells positive for both HAP1 with calretinin and HAP1with calbindin are indicated by double arrowheads. HAP1 single positive cell is indicated by arrowhead. Scale bar =50μm (A-L).

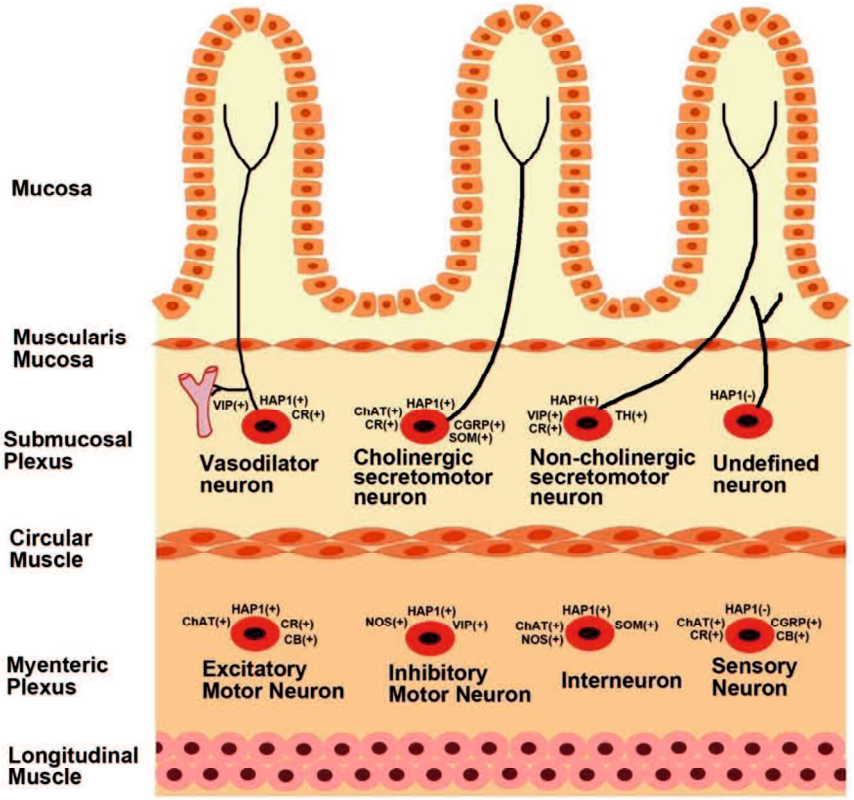

Fig. 26 Simplified schematic representing two layers of the small intestine in the enteric nervous system. Differentiated cells (vasodilator and secretomotor neurons) expressing the neurotransmitters are showed in the submucosal plexus where HAP1 is present in all the vasodilator and secretomotor neurons except some undefined neurons. ChAT, choline acetyltransferase; NO, nitric oxide synthases; VIP, vasoactive intestinal peptide; CR, calretinin; CB, calbindin; SP, substance P; SOM, somatostatin; CGRP, calcitonin gene-related peptide

Table 3. Co-expression ratios of HAP1 with different neuronal marker in the submucosal plexus in jejunum of mouse and rat

Relationship of HAP1 with different marker (ratio%)		Mouse	Rat
Marker	Interrelations		
ChAT	HAP1/ChAT	100.00±0.0	100.00±0.0
	ChAT/HAP1	43.75±2.4	45.75±2.9
CGRP	HAP1/CGRP	100.00±0.0	100.00±0.0
	CGRP/HAP1	41.25±1.4	44.75±1.25
Somatostatin	HAP1/SOM	100.00±0.0	100.00±0.0
	SOM/HAP1	27.5±1.5	31.25±2.2
VIP	HAP1/VIP	100.00±0.0	100.00±0.0
	VIP/HAP1	54.25±2.2	53.25±2.3
NOS	HAP1/NOS	100.00±0.0	100.00±0.0
	NOS/HAP1	1.75±0.25	9.25±0.8
TH	HAP1/TH	100.00±0.0	-
	TH/HAP1	22.25±1.3	-
Calretinin	HAP1/Calretinin	100.00±0.0	100.00±0.0
	Calretinin/HAP1	93.5±1.2	92.25±1.3
Calbindin	HAP1/Calbindin	100.00±0.0	100.00±0.0
	Calbindin/HAP1	33.5±1.8	42.05±2.3

ChAT, Choline acetyl transferase; CGRP, Calcitonin gene-related protein; TH, Tyrosine hydroxylase; VIP, Vasoactive Intestinal Peptide; NOS, Nitric acid synthase; SOM, Somatostatin; Values represent the mean ± SEM (n = 4)

Chapter VI

General summary and Conclusion

Our present study is the first to clarify the expression HAP1 in both myenteric and submucosal plexus of the ENS. Our current result shows that HAP1 is highly expressed in the ENS throughout the gastrointestinal tract. HAP1-immunoreactivity is present in excitatory motor neurons, inhibitory motor neurons and in interneurons except sensory neurons in the myenteric plexus. Our results may suggest the importance of HAP1 in regulation of both excitatory and inhibitory motor neuron or interneuron functions. It is possible that due to lack of putative STB/HAP1 protectivity, the sensory neurons (Dogiel type II) might be more vulnerable to neurodegeneration in different stresses. HAP1 could be used as a potential novel neurochemical marker for Dogiel type I neurons.

Our second study is also the first to examine the expression HAP1 in the Meisner's plexus of ENS. We have clarified the detailed expression and distribution of HAP1 in the submucosal plexus of enteric nervous system of mice and compared it with the rat gastrointestinal tract. We found that HAP1 was uniformly distributed in the Meissner's plexus and almost all the cholinergic secretomotor neurons and vasodilator neurons express HAP1. Our current study is the first to clarify that HAP1 is expressed in all type of cells in submucosal plexus. The current study might reflect the involvement of HAP1 in modulation of the secretomotor and vasodilator functions of submucosal neurons.

As HAP1 is present in all the cells of ENS except sensory neurons, these suggest that HAP1 can modulate the functions of enteric neurons especially motility functions. Furthermore, the ENS can act as a potential portal for pathogenesis of neurogenerative

disorders. ENS is also a neurodegenerative target in these disorders. It is possible that individuals with lower HAP1 expression or containing mutated HAP1 in enteric neurons might be vulnerable to different neurodegenerative disorders. Changes in the composition of gut microbiota in the GIT linked with the pathogenesis of neurological disorders in ENS and CNS. The microbiota/gut-and-brain axis is an emerging and widely accepted concept. Role of HAP1 in the microbiota-gut-brain axis in relation to neurodegenerative disorders might be an interesting area for future research. STB/HAP1 might affect the colonization of microbiota in intestine and consequently affect the neurodegeneration in ENS and CNS. Our current results lay a basic foundation for future studies that seek to clarify the physiological and pathological roles of HAP1 in the ENS which might provide new insights and shed light on yet-to-be-uncovered new diagnostic or therapeutic applications for neurodegenerative diseases including ALS, Alzheimer's disease, Parkinson's disease, Huntington's disease, and SBMA. In Japan and all over the world, the annual crude prevalence and incidence rates of neurodegenerative diseases are increasing in an alarming rate. Our results plausibly able to explain the answer that HAP1 protection might be the key to protect enteric autonomic/motor neurons and lack of HAP1 protection might make them more vulnerable to neurodegeneration. Our data also may create an opportunity to explore new diagnostic/therapeutic paradigms of certain neurodegenerative diseases utilizing tissues from the gastrointestinal tract.

Chapter VII

List of Abbreviations

ALS, amyotrophic Lateral Sclerosis
AR, androgen receptor
5-HT, 5-hydroxytryptamine
CB, calbindin
CGRP, calcitonin gene-related peptide
ChAT, choline acetyltransferase
CM, circular muscle
CR, calretinin
CNS, central nervous system
DRG, dorsal root ganglion
ENS, enteric nervous system
EPAN, extrinsic primary afferent neurons
GFAP, glial fibrillary acidic protein
GIT, gastrointestinal Tract
HAP1, huntingtin associated protein 1
Htt, huntingtin
Iba1, ionized calcium binding-adapter molecule
ICC, interstitial cell of Cajal
ICC-MY, interstitial cell of Cajal in the myenteric plexus
IgG, immunoglobulin G
IPAN, intrinsic primary afferent neurons
LM, longitudinal muscle
M, mucosa
MM, muscularis mucosae
NDS, normal donkey serum
NeuN, neuronal nuclei
NOS, nitric oxide synthases
PB, phosphate buffer
PBS, phosphate buffer saline
PBST-NDS, PBS containing 0.3% Triton X-100 and 0.05% NDS
PolyQ, polyglutamine
SBMA, spinal and bulbar muscular atrophy
SCA, spinocerebellar ataxia
S, serosa
SM, submucosa
SOM, somatostatin
SP, substance P
STB, stigmoid body
TBST, tris-buffer saline with 0.1 % Tween
TRPV1, transient receptor potential vanilloid 1
TH, tyrosine hydroxylase
VPAC1, vasoactive intestinal polypeptide receptor 1
VIP, vasoactive intestinal peptide

Chapter VIII

Acknowledgments

This dissertation work was carried out at the Laboratory of Neuroanatomy, The Graduate School of Medicine, Yamaguchi University. It is my pleasure to thank those people who made my study and research possible through their intensive efforts and participation.

First and foremost, I would like to thank my supervisor, Prof. Dr. Shinoda Koh and co-supervisors, Dr. Nabiul Islam for their excellent knowledge, guidance, wisdom, unfaltering enthusiasm and patience. I am indebted to them for their continuous help and encouragement throughout the years not only in the academic aspects but also in my daily life. I am greatly thankful to all my teachers, Mir Rubayet Jahan, Akie Yanai, Kanako Nozaki and Koh-hei Masumoto for their valuable comments, suggestions and guidance that made my preparation of this dissertation a reality.

I would like to acknowledge the technical expertise of the Institute for Biomedical Research and Education, Yamaguchi University Science Research Center. I would like to extend our gratitude to Ms. Takahashi, Mr. Chikahisa Matsuo, and Mr. Jun Oba for their administrative assistance. This work was supported by Grants-in-Aid for Scientifc Research of the Japan Society for the Promotion of Science (KAKENHI Grant Numbers 18K15006, 20K16108 to MNI, and 19K02318 to AY) and Grants-in-Aid for Basic Research of Fujii Setsuro Memorial Foundation (to KS). Finally, I am highly grateful to the Rotary Yoneyama Memorial Foundation and Ube Rotary Club, Japan, for sponsoring my study by providing me the Rotary Yoneyama Scholarship.

Chapter IX

References

Anlauf M, Schäfer MKH, Eiden L, Weihe E (2003) Chemical coding of the human gastrointestinal nervous system: Cholinergic, VIPergic, and catecholaminergic phenotypes. J Comp Neurol 459:90–111. https://doi.org/10.1002/cne.10599

Avetisyan M, Schill EM, Heuckeroth RO (2015) Building a second brain in the bowel. J Clin Invest 125:899-907. https://doi.org/10.1172/JCI76307

Banks MR, Farthing MJ, Robberecht P, Burleigh DE (2005) Antisecretory actions of a novel vasoactive intestinal polypeptide (VIP) antagonist in human and rat small intestine. British Journal of Pharmacology 144:994-1001. https://doi.org/10.1038/sj.bjp.0706128

Björkqvist M, Wild EJ, Thiele J, Silvestroni A, Andre R, Lahiri N, Raibon E, Lee RV, Benn CL, Soulet D, Magnusson A, Woodman B, Landles C, Pouladi MA, Hayden MR, Khalili-Shirazi A, Lowdell MW, Brundin P, Bates GP, Leavitt BR, Möller T, Tabrizi SJ (2008) A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease. J Exp Med 205:1869–1877. https://doi.org/10.1084/jem.20080178

Brehmer A, Schrödl F, Neuhuber W (1999) Morphological classifications of enteric neurons - 100 years after Dogiel. Anat. Embryol. (Berl). 200:125–135. https://doi.org/10.1007/s004290050267

Britton E, McLaughlin JT (2013) Ageing and the gut. Proc Nutr Soc 72(1):173-7. https://doi.org/10.1017/S0029665112002807

Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C (2012) Gamma-aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol 113:411–41. https://doi.org/10.1111/j.1365-2672.2012.05344.x

Bornstein JC, Costa M, Furness JB (1986) Synaptic inputs to immunohistochemically identified neurones in the submucous plexus of the guinea-pig small intestine. Journal of Physiology 381:465-482. https://doi.org/10.1113/jphysiol.1986.sp016339

Bornstein JC, Costa M, Furness JB (1988) Intrinsic and extrinsic inhibitory synaptic inputs to submucous neurones of the guinea-pig small intestine. Journal of Physiology 398:371-390. https://doi.org/10.1113/jphysiol.1988.sp017048

Bornstein JC & Furness JB (1988) Correlated electrophysiological and histochemical studies of submucous neurons and their contribution to understanding enteric neural circuits. J Auton Nerv Syst 25:1–13. https://doi.org/10.1016/0165-1838(88)90002-1

Brookes SJH (2001) Classes of enteric nerve cells in the guinea-pig small intestine. Anat Rec 262:58–70. https://doi.org/10.1002/1097-0185(20010101)262:1<58::AID-AR1011>3.0.CO;2-V

Brehmer A (2006) Structure of enteric neurons. Adv Anat 186:1–95

Carabotti M, Scirocco A, Maselli MA, Severi C (2015) The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol 28:203-209

Castillo-Álvarez F, Marzo-Sola ME (2019) Role of the gut microbiota in the development of various neurological diseases. Neurologia S0213-4853(19)30082-9. https://doi.org/10.1016/j.nrl.2019.03.017

Cajal SRY (1911) Histologie du système nerveux de l'homme et des vertébrés. Maloine, Paris

Chalazonitis A, Rao M (2018) Enteric nervous system manifestations of neurodegenerative disease. Brain Res. 1693:207-213. https://doi.org/10.1016/j.brainres.2018.01.011

Chan EY, Nasir J, Gutekunst CA, Coleman S, Maclean A, Maas A, Metzler M, Gertsenstein M, Ross CA, Nagy A, Hayden MR (2002) Targeted disruption of Huntingtin-associated protein-1 (Hap1) results in postnatal death due to depressed feeding behavior. Hum Mol Genet 11:945–959. https://doi.org/10.1093/hmg/11.8.945

Chino Y, Fujimura M, Kitahama K, Fujimiya M (2002) Colocalization of NO and VIP in neurons of the submucous plexus in the rat intestine. Peptides 23:2245–2250. https://doi.org/10.1016/s0196-9781(02)00264-4

Cox HM, Rudolph A, Gschmeissner S (1994) Ultrastructural co-localization of neuropeptide Y and vasoactive intestinal polypeptide in neurosecretory

Chaumette T, Lebouvier T, Aubert P, Lardeux B, Qin C, Li Q, Accary D, Bézard E, Bruley des Varannes S, Derkinderen P, Neunlist M (2009) Neurochemical plasticity in the enteric nervous system of a primate animal model of experimental Parkinsonism. Neurogastroenterol Motil 21:215–222. https://doi.org/10.1111/j.1365-2982.2008.01226.x

Chen X, Xin N, Pan Y, Zhu L, Yin P, Liu Q, Yang W, Xu X, Li S, Li XJ (2020) Huntingtin-Associated Protein 1 in Mouse Hypothalamus Stabilizes Glucocorticoid Receptor in Stress Response. Front Cell Neurosci 14:125. https://doi.org/10.3389/fncel.2020.00125

Christofi FL, Wunderlich J, Yu JG, Wang YZ, Xue J, Guzman J, Javed N, Cooke H (2004) Mechanically evoked reflex electrogenic chloride secretion in rat distal colon is triggered by endogenous nucleotides acting at P2Y1, P2Y2, and P2Y4 receptors. The Journal of Comparative Neurology 469:16-36. https://doi.org/10.1002/cne.10961

Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13:701-12. doi: 10.1038/nrn3346. Epub 2012 Sep 12. https://doi.org/10.1038/nrn3346

Dragatsis I, Zeitlin S DP (2004) Huntingtin-associated protein 1 (Hap1) mutant mice bypassing the early postnatal lethality are neuroanatomically normal and fertile but display growth retardation. Hum Mol Genet 13:3115–3125. https://doi.org/10.1093/hmg/ddh328

De Jonge F, Van Nassauw L, De Man JG, De Winter BY, Van Meir F, Depoortere I, Peeters TL, Pelckmans PA, Van Marck E, Timmermans J-P (2003b) Effects of Schistosoma mansoni infection on somatostatin and somatostatin receptor 2A expression in mouse ileum. Neurogastroenterol Motil 15:149–159. ttps://doi.org/10.1046/j.1365-2982.2003.00400.x

Dragatsis I, Dietrich P, Zeitlin S (2000) Expression of the Huntingtin-associated protein 1 gene in the developing and adult mouse. Neurosci Lett 282:37–40. https://doi.org/10.1016/S0304-3940(00)00872-7

Endres K & Schäfer KH (2018) Influence of Commensal Microbiota on the Enteric Nervous System and Its Role in Neurodegenerative Diseases. Journal of innate immunity, 10: 172–180. https://doi.org/10.1159/000488629.

Evans RJ, Jiang MM, Surprenant A (1994) Morphological properties and projections of electrophysiologically characterized neurons in the guinea-pig submucosal plexus. Neuroscience 59:1093-1110. https://doi.org/10.1016/0306-4522(94)90308-5

Fleming MA, Ehsan L, Moore SR, Levin DE (2020) The Enteric Nervous System and Its Emerging Role as a Therapeutic Target. Gastroenterol. Res Pract https://doi.org/10.1155/2020/8024171

Fujinaga R, Kawano J, Matsuzaki Y, Kamei K, Yanai A, Sheng Z, Tanaka M, Nakahama K, Nagano M, Shinoda K (2004) Neuroanatomical distribution of huntingtin-associated protein 1-mRNA in the male mouse brain. J Comp Neurol 478:88–109. https://doi.org/10.1002/cne.20277

Fujinaga R, Yanai A, Nakatsuka H, Yoshida K, Takeshita Y, Uozumi K, Zhao C,

Hirata K, Kokubu K, Nagano M, Shinoda K (2007) Anti-human placental antigen complex X-P2 (hPAX-P2) anti-serum recognizes C-terminus of huntingtin-associated protein 1A common to 1B as a determinant marker for the stigmoid body. Histochem Cell Biol 128:335–348. https://doi.org/10.1007/s00418-007-0315-5

Fujinaga R, Takeshita Y, Uozumi K, Yanai A, Yoshioka K, Kokubu K, Shinoda K (2009) Microtubule-dependent formation of the stigmoid body as a cytoplasmic inclusion distinct from pathological aggresomes. Histochem Cell Biol 132:305–318. https://doi.org/10.1007/s00418-009-0618-9

Fung C, Vanden Berghe P (2020) Functional circuits and signal processing in the enteric nervous system. Cell Mol Life Sci 77:4505-4522. https://doi:10.1007/s00018-020-03543-6

Foong JPP, Tough IR, Cox HM, Bornstein JC (2014) Properties of cholinergic and non-cholinergic submucosal neurons along the mouse colon. Journal

Fung C & Vanden Bergh P (2020) Functional circuits and signal processing in the enteric nervous system. Cellular and molecular life sciences 77:4505–4522. https://doi.org/10.1007/s00018-020-03543-6

Furness JB, Alex G, Clark MJ, Lal VV (2003) Morphologies and projections of defined classes of neurons in the submucosa of the guinea-pig small intestine. The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology, 272: 475–483. doi: 10.1002/ar.a.10064

Furness JB (2000) Types of neurons in the enteric nervous system. J Auton Nerv Syst 81:87–96. https://doi.org/10.1016/S0165-1838(00)00127-2

Furness JB (2006) The organisation of the autonomic nervous system: Peripheral connections. Auton. Neurosci. Basic Clin. 130:1–5. https://doi.org/10.1016/j.autneu.2006.05.003

Furness JB (2012) The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 9:286–294. https://doi.org/10.1038/nrgastro.2012.32

Furness JB, Callaghan BP, Rivera LR, Cho HJ (2014) The enteric nervous system and gastrointestinal innervation: Integrated local and central control. Adv Exp Med Biol 817:39–71. https://doi.org/10.1007/978-1-4939-0897-4_3

Furness JB, Costa M (1980) Types of nerves in the enteric nervous system. Neuroscience 5:1–20. https://doi: 10.1016/0306-4522(80)90067-6. PMID: 6154268.

Furness JB, Kunze WA, Bertrand PP, Clerc N, Bornstein JC (1998) Intrinsic primary afferent neurons of the intestine. Prog Neurobiol 54:1–18. https://doi.org/10.1016/S0301-0082(97)00051-8

Furness JB, Robbins HL, Xiao J, Stebbing MJ, Nurgali K (2004) Projections and chemistry of Dogiel type II neurons in the mouse colon. Cell Tissue Res 317:1–12. https://doi.org/10.1007/s00441-004-0895-5

Gershon, M. The Second Brain. (Harper Collins, 1999)

Gutekunst CA, Li SH, Yi H, Ferrante RJ, Li XJ, Hersch SM (1998) The cellular and subcellular localization of huntingtin-associated protein 1 (HAP1): Comparison with huntingtin in rat and human. J Neurosci 18:7674–7686. https://doi.org/10.1523/jneurosci.18-19-07674.1998

Girotti, P. A., Misawa, R., Palombit, K., Mendes, C. E., Bittencourt, J. C., & Castelucci, P. (2013). Differential effects of undernourishment on the differentiation and maturation of rat enteric neurons. Cell and tissue research,

353(3), 367–380. https://doi.org/10.1007/s00441-013-1620-z

Heuckeroth RO (2018) Hirschsprung disease - Integrating basic science and clinical medicine to improve outcomes. Nat. Rev. Gastroenterol. Hepatol. 15:152–167. https://doi.org/10.1038/nrgastro.2017.149

Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y (1998) Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science. 279(5350):577-80. https://doi: 10.1126/science.279.5350.577

Hansen MB, Witte AB (2008) The role of serotonin in intestinal luminal sensing and secretion. Acta Physiol 193:311–323. https://doi.org/10.1111/j.1748-1716.2008.01870.x

Harrington, A. M., Hutson, J. M., & Southwell, B. R. (2005). Immunohistochemical localization of substance P NK1 receptor in guinea pig distal colon. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society, 17(5), 727–737. https://doi.org/10.1111/j.1365-2982.2005.00680.x

Hens J, Schrödl F, Brehmer A, Adriaensen D, Neuhuber W, Scheuermann DW, Schemann M, Timmermans JP (2000). Mucosal projections of enteric neurons in the porcine small intestine. The Journal of comparative neurology, 421(3), 429–436.

Hens J, Vanderwinden JM, De Laet MH, Scheuermann DW, Timmermans JP (2001) Morphological and neurochemical identification of enteric neurons with mucosal projections in the human small intestine. Journal of Neurochemistry 76:464-471.

Islam MN, Fujinaga R, Yanai A, Jahan MR, Takeshita Y, Kokubu K, Shinoda K (2012) Characterization of the “sporadically lurking HAP1-immunoreactive (SLH) cells” in the hippocampus, with special reference to the expression of

steroid receptors, GABA, and progenitor cell markers. Neuroscience 210:67–81. https://doi.org/10.1016/j.neuroscience.2012.02.029

Islam MN, Takeshita Y, Yanai A, Imagawa A, Jahan MR, Wroblewski G, Nemoto J, Fujinaga R, Shinoda K (2017) Immunohistochemical analysis of huntingtin-associated protein 1 in adult rat spinal cord and its regional relationship with androgen receptor. Neuroscience 340:201–217. https://doi.org/10.1016/j.neuroscience.2016.10.053

Islam MN, Maeda N, Miyasato E, Jahan MR, Tarif AMM, Ishino T, Nozaki K, Masumoto KH, Yanai A, Shinoda K (2020a) Expression of huntingtin-associated protein 1 in adult mouse dorsal root ganglia and its neurochemical characterization in reference to sensory neuron subpopulations. IBRO Rep. 9:258-269. https://doi.org/10.1016/j.ibror.2020.10.001

Islam MN, Sakimoto Y, Jahan MR, Ishida M, Tarif AMM, Nozaki K, Masumoto KH, Yanai A, Mitsushima D, Shinoda K (2020b) Androgen affects the dynamics of intrinsic plasticity of pyramidal neurons in the CA1 hippocampal subfield in adolescent male rats. Neuroscience 440:15-29. https://doi.org/10.1016/j.neuroscience.2020.05.025

Islam MN, Sakimoto Y, Jahan MR, Miyasato E, Tarif AMM, Nozaki K, Masumoto KH, Yanai A, Mitsushima D, Shinoda K (2021) Androgen affects the inhibitory avoidance memory by primarily acting on androgen receptor in the brain in adolescent male rats. Brain Sci. 11:239. doi: 10.3390/brainsci11020239. https://doi.org/10.3390/brainsci11020239

Page KJ, Potter L, Aronni S, Everitt BJ, Dunnett SB (1998) The expression of Huntingtin-associated protein (HAP1) mRNA in developing, adult and ageing rat CNS: implications for Huntington's disease neuropathology. Eur J Neurosci 10:1835-45. https://doi.org/10.1046/j.1460-9568.1998.00185.x

Jahan MR, Kokubu K, Islam MN, Matsuo C, Yanai A, Wroblewski G, Fujinaga R, Shinoda K (2015) Species differences in androgen receptor expression in the

medial preoptic and anterior hypothalamic areas of adult male and female rodents. Neuroscience 284:943–961. https://doi.org/10.1016/j.neuroscience.2014.11.003

Kamei K, Matsuzaki Y, Hanada K, Nagano M, Nakahama K, Shinoda K (2001) Distribution of mRNA in the rat brain. Acta Anat Nippon 76(Suppl): A-91

Khan TK, Alkon DL (2010) Early diagnostic accuracy and pathophysiologic relevance of an autopsy-confirmed Alzheimer's disease peripheral biomarker. Neurobiol Aging 31:889–900. https://doi.org/10.1016/j.neurobiolaging.2008.07.010

Kim S, Kwon SH, Kam TI, Panicker N, Karuppagounder SS, Lee S, Lee JH, Kim WR, Kook M, Foss CA, Shen C, Lee H, Kulkarni S, Pasricha PJ, Lee G, Pomper MG, Dawson VL, Dawson TM, Ko HS (2019) Transneuronal Propagation of Pathologic α-Synuclein from the Gut to the Brain Models Parkinson's Disease. Neuron. 103(4):627-641.e7. https://doi.org/10.1016/j.neuron.2019.05.035

Koga M, Fujinaga R, Shinoda K. (2002) Interaction between HAP1 and polyglutaine protein in vitro: 57th Chugoku-Shikoku Conference of Japanese Association of Anatomists. November 9, Yonago, Japan p 13 (Abstr).

Kuwahara A, Matsuda K, Kuwahara Y, Asano S, Inui T, Marunaka Y (2020) Microbiota-gut-brain axis: enteroendocrine cells and the enteric nervous system form an interface between the microbiota and the central nervous system. Biomed Res 41:199-216. https://doi.org/10.2220/biomedres.41.199

Kunze WAA, Furness JB (1999) The enteric nervous system and regulation of intestinal motility. Annu Rev Physiol 61:117–142. https://doi.org/10.1146/annurev.physiol.61.1.117

Kordasti S, Sapnara M, Thomas EA, Lindstrom E, Forsman M, Bornstein JC, Sjovall H (2006) Effects of cholera toxin on the potential difference and motor responses induced by distension in the rat proximal small intestine in vivo. American

Journal of Physiology Gastrointestinal and Liver Physiology 290:G948-958. https://doi.org/10.1152/ajpgi.00267.2005

Krueger D, Michel K, Zeller F, Demir IE, Ceyhan GO, Slotta-Huspenina J, Schemann M (2016) Neural influences on human intestinal epithelium in vitro. Journal of Physiology 594:357-372. https://doi.org/10.1113/JP271493

Kuwahara A, Matsuda K, Kuwahara Y, Asano S, Inui T, Marunaka Y (2020) Microbiota-gut-brain axis: enteroendocrine cells and the enteric nervous system form an interface between the microbiota and the central nervous system. Biomed Res 41:199–216. https://doi.org/10.2220/biomedres.41.199

Lai NY, Musser MA, Pinho-Ribeiro FA, Baral P, Jacobson A, Ma P, Potts DE, Chen Z, Paik D, Soualhi S, Yan Y, Misra A, Goldstein K, Lagomarsino VN, Nordstrom A, Sivanathan KN, Wallrapp A, Kuchroo VK, Nowarski R, Starnbach MN, Shi H, Surana NK, An D, Wu C, Huh JR, Rao M, Chiu IM (2020) Gut-innervating nociceptor neurons regulate Peyer's patch microfold cells and SFB levels to mediate salmonella host defense. Cell 180:33-49.e22. https://doi.org/10.1016/j.cell.2019.11.014

Lebouvier T, Chaumette T, Paillusson S, Duyckaerts C, Bruley des Varannes S, Neunlist M, Derkinderen P (2009) The second brain and Parkinson’s disease. Eur. J. Neurosci. 30:735–741. https://doi.org/10.1111/j.1460-9568.2009.06873.x

Li SH, Hosseini SH, Gutekunst CA, Hersch SM, Ferrante RJ, Li XJ (1998) A human HAP1 homologue: Cloning, expression, and interaction with huntingtin. J Biol Chem 273:19220–19227. https://doi.org/10.1074/jbc.273.30.19220

Li SH, Yu ZX, Li CL, Nguyen HP, Zhou YX, Deng C, Li XJ (2003) Lack of huntingtin-associated protein-1 causes neuronal death resembling hypothalamic degeneration in Huntington’s disease. J Neurosci 23:6956–6964. https://doi.org/10.1523/jneurosci.23-17-06956.2003

Li T, Li S, Gao X, Cai Q, Li XJ (2019) Expression and Localization of Huntingtin-Associated Protein 1 (HAP1) in the Human Digestive System. Dig Dis Sci 64:1486–1492. https://doi.org/10.1007/s10620-018-5425-5

Li XJ, Li SH, Sharp AH, Nucifora FC Jr, Schilling G, Lanahan A, Worley P, Snyder SH, Ross CA (1995) A huntingtin-associated protein enriched in brain with implications for pathology. Nature 378:398–402. https://doi.org/10.1038/378398a0

Li XJ, Sharp AH, Li SH, Dawson TM, Snyder SH, Ross CA (1996) Huntingtin-associated protein (HAP1): Discrete neuronal localizations in the brain resemble those of neuronal nitric oxide synthase. Proc Natl Acad Sci U S A 93:4839–4844. https://doi.org/10.1073/pnas.93.10.4839

Li ZS, Pham TD, Tamir H, Chen JJ, Gershon MD (2004) Enteric Dopaminergic Neurons: Definition, Developmental Lineage, and Effects of Extrinsic Denervation. J Neurosci 24:1330–1339. https://doi.org/10.1523/JNEUROSCI.3982-03.2004

Liao M, Shen J, Zhang Y, Li SH, Li XJ, Li H (2005) Immunohistochemical localization of Huntingtin-associated protein 1 in endocrine system of the rat. J Histochem Cytochem 53:1517–1524. https://doi.org/10.1369/jhc.5A6662.2005

Lin YF, Xu X, Cape A, Li S, Li XJ (2010) Huntingtin-associated protein-1 deficiency in orexin-producing neurons impairs neuronal process extension and leads to abnormal behavior in mice. J Biol Chem 285:15941–15949. https://doi.org/10.1074/jbc.M110.107318

Liu Q, Cheng S, Yang H, Zhu L, Pan Y, Jing L, Tang B, Li S, Li XJ (2020) Loss of Hap1 selectively promotes striatal degeneration in Huntington disease mice. Proc Natl Acad Sci U S A 117:20265–20273. https://doi.org/10.1073/PNAS.2002283117

Lomax AE, Furness JB (2000) Neurochemical classification of enteric neurons in the

guinea-pig distal colon. Cell Tissue Res 302:59–72. https://doi.org/10.1007/s004410000260

Margolis KG, Gershon MD, Bogunovic M (2016) Cellular organization of neuroimmune interactions in the gastrointestinal tract. Trends Immunol 37(7):487–501. https://doi.org/10.1016/j. it.2016.05.003

Masliukov PM, Moiseev K, Budnik AF, Nozdrachev AD, Timmermans JP (2017) Development of Calbindin- and Calretinin-Immunopositive Neurons in the Enteric Ganglia of Rats. Cell Mol Neurobiol 37:1257–1267. https://doi.org/10.1007/s10571-016-0457-x

Mawe GM, Schemann M, Wood JD, Gershon MD (1989) Immunocytochemical analysis of potential neurotransmitters present in the myenteric plexus and muscular layers of the corpus of the guinea pig stomach. Anat Rec 224:431–42. https://doi.org/10.1002/ar.1092240312

Mongardi Fantaguzzi C, Thacker M, Chiocchetti R, Furness JB (2009). Identification of neuron types in the submucosal ganglia of the mouse ileum. Cell and tissue research, 336, 179–189. https://doi.org/10.1007/s00441-009-0773-2

Mazzoni M, Caremoli F, Cabanillas L, de Los Santos J, Million M, Larauche M, Clavenzani P, De Giorgio R, Sternini C (2020) Quantitative analysis of enteric neurons containing choline acetyltransferase and nitric oxide synthase immunoreactivities in the submucosal and myenteric plexuses of the porcine colon. Cell Tissue Res. https://doi.org/10.1007/s00441-020-03286-7

Metzger S, Rong J, Nguyen HP, Cape A, Tomiuk J, Soehn AS, Propping P, Freudenberg-Hua Y, Freudenberg J, Tong L, Li SH, Li XJ, Riess O (2008) Huntingtin-associated protein-1 is a modifier of the age-at-onset of Huntington's disease. Hum Mol Genet 17:1137-1146. https://doi.org/10.1093/hmg/ddn003

Mitsui R (2009) Characterisation of calcitonin gene-related peptide-immunoreactive neurons in the myenteric plexus of rat colon. Cell Tissue Res 337:37–43.

https://doi.org/10.1007/s00441-009-0798-6

Murphy EM, Defontgalland D, Costa M, Brookes SJ, Wattchow DA (2007) Quantification of subclasses of human colonic myenteric neurons by immunoreactivity to Hu, choline acetyltransferase and nitric oxide synthase. Neurogastroenterol Motil 19:126–134. https://doi.org/10.1111/j.1365-2982.2006.00843.x

Mourad FH, Nassar CF (2000) Effect of vasoactive intestinal polypeptide (VIP) antagonism on rat jejunal fluid and electrolyte secretion induced by cholera and Escherichia coli enterotoxins. Gut 47:382-386. https://doi.org/10.1136/gut.47.3.382

Niu SN, Huang ZB, Wang H, Rao XR, Kong H, Xu J, Li XJ, Yang C, Sheng GQ (2011) Brainstem Hap1-Ahi1 is involved in insulin-mediated feeding control. FEBS Lett 585:85–91. https://doi.org/10.1016/j.febslet.2010.11.059

Noorian AR, Taylor GM, Annerino DM, Greene JG (2011) Neurochemical phenotypes of myenteric neurons in the rhesus monkey. J Comp Neurol 519:3387–3401. https://doi.org/10.1002/cne.22679

O'Neill OM, Johnston BT, Coleman HG (2013) Achalasia: A review of clinical diagnosis, epidemiology, treatment and outcomes. World J. Gastroenterol. 19:5806–5812. https://doi.org/10.3748/wjg.v19.i35.5806

Obata Y, Castano A, Boeing S, Bon-Frauches AC, Fung C, Fallesen T, de Aguero MG, Yilmaz B, Lopes R, Huseynova A, Horswell S, Maradana MR, Boesmans W, Vanden Berghe P, Murray AJ, Stockinger B, Macpherson AJ, Pachnis V (2020) Neuronal programming by microbiota regulates intestinal physiology. Nature 578(7794):284–289. https://doi. org/10.1038/s41586-020-1975-8

Pham TD, Gershon MD, Rothman TP (1991) Time of origin of neurons in the murine enteric nervous system: sequence in relation to phenotype. J Comp Neurol 314:789–798. https://doi.org/10.1002/cne.903140411

Phillips RJ, Powley TL (2007) Innervation of the gastrointestinal tract: patterns of aging. Auton Neurosci 136:1-19. https://doi.org/10.1016/j.autneu.2007.04.005

Pompolo S, Furness JB (1998) Quantitative analysis of inputs to somatostatin-immunoreactive descending interneurons in the myenteric plexus of the guinea-pig small intestine. Cell Tissue Res 294:219–226. https://doi.org/10.1007/s004410051171

Porter AJ, Wattchow DA, Brookes SJH, Costa M (2002) Cholinergic and nitrergic interneurones in the myenteric plexus of the human colon. Gut 51:70–75. https://doi.org/10.1136/gut.51.1.70

Prigge JR, Schmidt EE (2007) HAP1 can sequester a subset of TBP in cytoplasmic inclusions via specific interaction with the conserved TBPCORE. BMC Mol Biol 8:. https://doi.org/10.1186/1471-2199-8-76

Qu ZD, Thacker M, Castelucci P, Bagyánszki M, Epstein ML, Furness JB (2008) Immunohistochemical analysis of neuron types in the mouse small intestine. Cell Tissue Res 334:147–161. https://doi.org/10.1007/s00441-008-0684-7

Riddle M, Martineau B, Peavey M, Tabin C (2018) Raising the Mexican Tetra Astyanax mexicanus for Analysis of Post-larval Phenotypes and Whole-mount Immunohistochemistry. J Vis Exp.142:10.3791/58972. https://doi: 10.3791/58972

Reed DE & Vanner SJ (2001). Converging and diverging cholinergic inputs from submucosal neurons amplify activity of secretomotor neurons in guinea-pig ileal submucosa. Neuroscience, 107, 685–696. https://doi.org/10.1016/s0306-4522(01)00392-x

Schneider J, Jehle EC, Starlinger MJ, Neunlist M, Michel K, Hoppe S, Schemann M (2001) Neurotransmitter coding of enteric neurons in the submucous plexus is changed in non-inflamed rectum of patients with Crohn's disease. Neurogastroenterology and Motility 13:255-264. https://doi.org/10.1046/j.1365-

2982.2001.00265.x

Sang Q YH (1998) The Identification and Chemical Coding of Cholinergic Neurons in the Small and Large Intestine of the Mouse. Anat Rec 251:185–199. https://doi.org/10.1002/(SICI)1097-0185(199806)251:2<185::AID-AR6>3.0.CO;2-Y

Sayegh AI, Ritter RC (2003) Morphology and distribution of nitric oxide synthase-, neurokinin-1 receptor-, calretinin-, calbindin-, and neurofilament-M-immunoreactive neurons in the myenteric and submucosal plexuses of the rat small intestine. Anat Rec - Part A Discov Mol Cell Evol Biol 271:209–216. https://doi.org/10.1002/ar.a.10024

Sharon G, Sampson TR, Geschwind DH, Mazmanian SK (2016) The Central Nervous System and the Gut Microbiome. Cell 167:915-932. https://doi.org/10.1016/j.cell.2016.10.027

Sheng G, Chang GQ, Lin JY, Yu ZX, Fang ZH, Rong J, Lipton SA, Li SH, Tong G, Leibowitz SF, Li XJ (2006) Hypothalamic huntingtin-associated protein 1 as a mediator of feeding behavior. Nat Med 12:526–533. https://doi.org/10.1038/nm1382

Sheng G, Xu X, Lin YF, Wang CE, Rong J, Cheng D, Peng J, Jiang X, Li SH, Li XJ (2008) Huntingtin-associated protein 1 interacts with Ahi1 to regulate cerebellar and brainstem development in mice. J Clin Invest 118:2785–2795. https://doi.org/10.1172/JCI35339

Shinoda K, Mori S, Ohtsuki T, Osawa Y (1992) An aromatase-associated cytoplasmic inclusion, the ?stigmoid body?, in the rat brain: I. Distribution in the forebrain. J Comp Neurol 322:360–376. https://doi.org/10.1002/cne.903220306

Shinoda K, Nagano M, Osawa Y (1993) An aromatase-associated cytoplasmic inclusion, the “stigmoid body,” in the rat brain: II. Ultrastructure (with a review of its history and nomenclature). J Comp Neurol 329:1–19.

https://doi.org/10.1002/cne.903290102

Smolilo DJ, Costa M, Hibberd TJ, Brookes SJH, Wattchow DA, Spencer NJ (2019) Distribution, projections, and association with calbindin baskets of motor neurons, interneurons, and sensory neurons in guinea-pig distal colon. J Comp Neurol 527:1140–1158. https://doi.org/10.1002/cne.24594

Suganya K, Koo BS (2020) Gut-Brain Axis: Role of Gut Microbiota on Neurological Disorders and How Probiotics/Prebiotics Beneficially Modulate Microbial and Immune Pathways to Improve Brain Functions. Int J Mol Sci 21:7551. https://doi.org/10.3390/ijms21207551

Swaminathan M, Fung C, Finkelstein DI, Bornstein JC, Foong JPP (2019) α-Synuclein Regulates Development and Function of Cholinergic Enteric Neurons in the Mouse Colon. Neuroscience 423:76–85. https://doi.org/10.1016/j.neuroscience.2019.10.029

Tarif AMM, Islam MN, Jahan MR, Yanai A, Nozaki K, Masumoto KH, Shinoda K (2021) Immunohistochemical expression and neurochemical phenotypes of huntingtin-associated protein 1 in the myenteric plexus of mouse gastrointestinal tract. Cell Tissue Res. 386(3): 533–558. https://doi.org/10.1007/s00441-021-03542-4

Takeshita Y, Fujinaga R, Zhao C, Yanai A, Shinoda K (2006) Huntingtin-associated protein 1 (HAP1) interacts with androgen receptor (AR) and suppresses SBMA-mutant-AR-induced apoptosis. Hum Mol Genet. 2006;15:2298-2312 https://doi.org/10.1093/hmg/ddl156

Takeshita Y, Fujinaga R, Kokubu K, Islam MN, Jahan MR, Yanai A, Kakizuka A, Shinoda K (2011) Interaction of ataxin-3 with huntingtin-associated protein 1 through Josephin domain. Neuroreport 22:232–238. https://doi.org/10.1097/WNR.0b013e32834505f4

Timmermans JP, Hens J, Adriaensen D (2001) Outer submucous plexus: an intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. The Anatomical Record 262:71-78.

Wasser CI, Mercieca EC, Kong G, Hannan AJ, McKeown SJ, Glikmann-Johnston Y, Stout JC (2020) Gut dysbiosis in Huntington's disease: associations among gut microbiota, cognitive performance and clinical outcomes. Brain Commun 2:fcaa110. https://doi.org/10.1093/braincomms/fcaa110

Uyttebroek L, Shepherd IT, Hubens G, Timmermans JP, Van Nassauw L (2013). Expression of neuropeptides and anoctamin 1 in the embryonic and adult zebrafish intestine, revealing neuronal subpopulations and ICC-like cells. Cell Tissue Res. 354:355–370. https://doi.org/10.1007/s00441-013-1685-8

Van Op den Bosch J, Lantermann K, Torfs P, Van Marck E, Van Nassauw L, Timmermans JP (2008) Distribution and expression levels of somatostatin and somatostatin receptors in the ileum of normal and acutely Schistosoma mansoni-infected SSTR2 knockout/lacZ knockin mice. Neurogastroenterol Motil 20:798–807. https://doi.org/10.1111/j.1365-2982.2008.01088.x

Vanden Berghe P, Coulie B, Tack J, Mawe GM, Schemann M, Janssens J (1999) Neurochemical coding of myenteric neurons in the guinea-pig antrum. Cell Tissue Res 297:81–90. https://doi.org/10.1007/s004410051335

Van der Burg JM, Winqvist A, Aziz NA, Maat-Schieman ML, Roos RA, Bates GP, Brundin P, Björkqvist M, Wierup N (2011) Gastrointestinal dysfunction contributes to weight loss in Huntington's disease mice. Neurobiol Dis 44:1–8. https://doi.org/10.1016/j.nbd.2011.05.006

Van Nassauw L, Hens J, Bogers J, Van Marck E, Timmermans J-P (2000) Retrograde DiI tracing of enteric neurons projecting to the mucosa in the murine small intestine. In: Krammer H-J, Singer MV (eds) Neurogastroenterology, from the basics to the clinics. Kluwer Academic, Dordrecht, pp 92–95

Vanner S, Surprenant A (1996) Neural refexes controlling intestinal microcirculation. Am J Physiol 271(2 Pt 1):G223–230. https://doi.org/10.1152/ajpgi.1996.271.2.G223

Vanner S, Macnaughton WK (2004) Submucosal secretomotor and vasodilator refexes. Neurogastroenterol Motil 16(Suppl 1):39–43. https://doi.org/10.1111/j.1743-3150.2004.00473.x

Wasser CI, Mercieca EC, Kong G, Hannan AJ, McKeown SJ, Glikmann-Johnston Y, Stout JC (2020) Gut dysbiosis in Huntington's disease: associations among gut microbiota, cognitive performance and clinical outcomes. Brain Commun 2:fcaa110. https://doi.org/10.1093/braincomms/fcaa110

Wattchow D, Brookes S, Murphy E, Carbone S, de Fontgalland D, Costa M (2008) Regional variation in the neurochemical coding of the myenteric plexus of the human colon and changes in patients with slow transit constipation. Neurogastroenterol Motil 20:1298–1305. https://doi.org/10.1111/j.1365-2982.2008.01165.x

Wroblewski G, Islam MN, Yanai A, Jahan MR, Masumoto KH, Shinoda K (2018) Distribution of HAP1-immunoreactive Cells in the Retrosplenial–retrohippocampal Area of Adult Rat Brain and Its Application to a Refined Neuroanatomical Understanding of the Region. Neuroscience 394:109–126. https://doi.org/10.1016/j.neuroscience.2018.10.029

Wong V, Blennerhassett M, Vanner S (2008) Electrophysiological and morphological properties of submucosal neurons in the mouse distal colon. Neurogastroenterology and Motility 20:725-734. https://doi.org/10.1111/j.1365-2982.2008.01117.x

Yanai A, Islam MN, Hayashi-Okada M, Jahan MR, Tarif AMM, Nozaki K, Masumoto KH, Shinoda K (2020). Immunohistochemical relationships of huntingtin-associated protein 1 with enteroendocrine cells in the pyloric mucosa of the rat stomach. Acta Histochem. 122:151650. https://doi:

10.1016/j.acthis.2020.151650.

Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, Nagler CR, Ismagilov RF, Mazmanian SK, Hsiao EY (2015) Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell 161:264–276. https://doi.org/10.1016/j.cell.2015.02.047

Young HM, Ciampoli D (1998) Transient expression of neuronal nitric oxide synthase by neurons of the submucous plexus of the mouse small intestine. Cell Tissue Res 291:395–401. https://doi.org/10.1007/s004410051009

Zetzmann K, Strehl J, Geppert C, Kuerten S, Jabari S, Brehmer A. (2018) Calbindin D28k-Immunoreactivity in Human Enteric Neurons. Int J Mol Sci. 19(1):194. https://doi.org/10.3390/ijms19010194

Zhu L, Song X, Tang J, Wu J, Ma R, Cao H, Ji M, Jing C, Wang Z (2013) Huntingtin-associated protein 1: A potential biomarker of breast cancer. Oncol Rep 29:1881–1887. https://doi.org/10.3892/or.2013.2303