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bHLH proneural genes as cell fate determinants of entero-endocrine cells, an evolutionarily conserved lineage sharing a common root with sensory neurons

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Abstract

Entero-endocrine cells involved in the regulation of digestive function form a large and diverse cell population within the intestinal epithelium of all animals. Together with absorptive enterocytes and secretory gland cells, entero-endocrine cells are generated by the embryonic endoderm and, in the mature animal, from a pool of endoderm derived, self-renewing stem cells. Entero-endocrine cells share many structural/functional and developmental properties with sensory neurons, which hints at the possibility of an ancient evolutionary relationship between these two cell types. We will survey in this article recent findings that emphasize the similarities between entero-endocrine cells and sensory neurons in vertebrates and insects, for which a substantial volume of data pertaining to the entero-endocrine system has been compiled. We will then report new findings that shed light on the specification and morphogenesis of entero-endocrine cells in *Drosophila*. In this system, presumptive intestinal stem cells (pISCs), generated during early metamorphosis, undergo several rounds of mitosis that produce the endocrine cells and stem cells (ISCs) with which the fly is born. Clonal analysis demonstrated that individual pISCs can give rise to endocrine cells expressing different types of peptides. Immature endocrine cells start out as unpolarized cells located basally of the gut epithelium; they each extend an apical process into the epithelium which establishes a junctional complex and apical membrane specializations contacting the lumen of the gut. Finally, we show that the *Drosophila* homolog of *ngn3*, a bHLH gene that defines the entero-endocrine lineage in mammals, is expressed and required for the differentiation of this cell type in the fly gut.

Graphical

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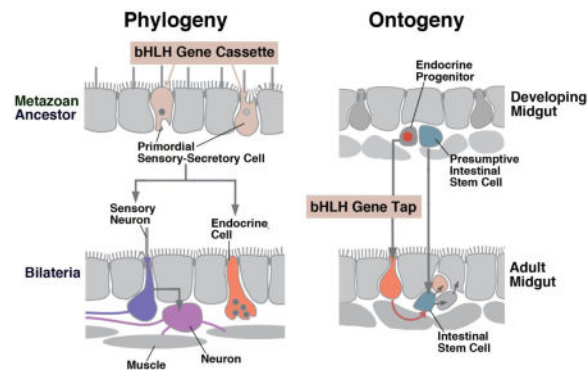
Keywords

entero-endocrine cell; neuron; development; lineage; neurogenin; Drosophila

Introduction

Sensory neurons and entero-endocrine cells: an evolutionary perspective

Sensory neurons and endocrine cells lining the intestinal epithelium share many functional, structural and developmental properties. Sensory neurons, in particular gustatory neurons, and entero-endocrine cells express the same or related types of receptors on their apical membrane (Rozengurt and Sternini, 2007; Mace et al., 2015; Gribble and Reimann, 2016); upon stimulation, transmitters are released basally which, in case of gustatory neurons, synaptically depolarize the target neurons, or, in entero-endocrine cells, reach their target tissues via the blood stream or local diffusion. The structural and functional similarity is reflected in highly conserved molecular pathways controlling the development of neurons and entero-endocrine cells, and may hint at a deep evolutionary relationship between the two cell systems at the base of the metazoa. Basal metazoa lacking a nervous system or muscular system, including porifera and placozoa, possess cells with the characteristics of endocrine or exocrine gland cells. For example, in placozoa, gland cells with secretory granules, expressing proteins that form part of the synaptic compexes in higher animals (synapsin, syntaxin, SNAP-24), as well as neuropeptides (FMRFamide), are integrated in the ciliated epithelium that forms the body wall (Smith et al., 2014; Fig.1A, B). Structurally similar secretory cells were described in larvae of the sponge *Amphimedon*, where they mount a Ca-response to environmental stimuli that is involved in larval settling and metamorphosis (Nakanishi et al., 2015). It is conceivable that such specialized epithelial cells, which have acquired sensory, neurosecretory and effector properties, could constitute the "protoneurons" that were present already in basal metazoans, and that gave rise to both neurons and endocrine cells in the bilaterian animals.

As schematically depicted in Fig.1C, both outer (ectodermally derived) and inner (endodermally derived) epithelia of bilaterians are accompanied by a layer of muscle cells and neurons. In the intestine, neurons form an enteric plexus (called stomatogastric or pharyngeal nervous system in many invertebrate taxa); secretory gland cells and entero-endocrine cells are integrated in the intestinal epithelium. Endocrine cells, enteric neurons,

and groups of neurons in the brain express a large number of phylogenetically related transmitters/hormones, called "brain-gut peptides", in addition to being interconnected by nerve fibers. Taken together, these elements form a complex integrated system, the "brain-gut axis", that controls feeding behavior and digestive functions, including motility of the gut, secretion of enzymes, and resorption and excretion of nutrients, ions and water (for recent review, see Latorre et al., 2016; Gribble and Reimann, 2016).

In the following, we will survey recent research data pertaining to the structure, function and development of the entero-endocrine system. Outside several vertebrate models, a good amount of knowledge has accumulated in various arthropods. The similarities that exist in enteroendocrine cells and sensory neurons in regard to their structure, expression of peptides/signaling pathways, and, in particular, the developmental mechanisms by which they are specified, are compatible with the idea of a shared origin of these cell types.

Comparison of the structure and function of the entero-endocrine system in insects and vertebrates

Entero-endocrine cells are specialized, endodermally derived cells with a cell body located basally, and a neck that reaches towards the luminal surface of the epithelium (Fig.2A3). According to recent serial electron microscopy studies (Bohorquez et al., 2014; 2015), entero-endocrine cells project elongated basal processes, so called neuropods, that interact with glial cells and neurons of the enteric plexus (Fig.2B). Neurons of the enteric plexus originate from the neural crest and migrate towards the gut; in a similar fashion, enteric (stomatogastric) neurons of insects are generated in the foregut ectoderm and undergo migration to reach their destination in the intestinal wall (Fig.2A1).

Endocrine cells possess two regulated pathways of secretion which are structurally defined by large dense core vesicles (LDCV) and synaptic-like microvesicles (SLMV; Rindi et al., 2004). Dense core vesicles have an electron-dense interior and measure 80–100nm; they are regularly associated with the storage and release of neuropeptides. Microvesicles resemble the small synaptic vesicles (20–40nm) releasing classical transmitters of neurons, such as acetyl choline, at the synaptic cleft. In enteroendocrine cells, both types of vesicles are targeted to the basal cell membrane, and released into the interstitial space surrounding enteric neurons/glia and capillaries, or, in case of insects, the open hemolymph space.

The cellular mechanisms controlling stimulus reception, vesicle trafficking and docking, as well as the released peptides themselves, are very similar in entero-endocrine cells and sensory neurons. Typical neuronal markers like N-CAM, synaptophysin, or vesicular monoamine transporter, are also found in entero-endocrine cells, where they perform the same or similar functions. Thus, the docking of vesicles, as well as the transport and re-uptake of transmitters utilize conserved molecular pathways. Importantly, also the gustatory G-protein-coupled receptors expressed in sensory neurons of the mouth cavity and tongue. Numerous members of the T1R family (sensing of sugars and L-amino acids) and T2R (bitter tastes) are expressed in the intestine, and could be assigned to numerous types of entero-endocrine cells (Rozengurt and Sternini, 2007). Similarly, signal transducers, including Gα-gustducin and transducing 2, were also found to be expressed in these cells. Stimulation of G-protein-coupled receptors results in an increase in intracellular calcium,

and functional studies confirmed that tastants applied to entero-endocrine-derived cell lines caused a Ca-spike, and led to a release of peptides, including cholecystokinin (CKK; from STC-1 cells stimulated with bitter tastants) and glucagon-like peptide (GLP-1; from GLUTag cells stimulated by sweet tastants) (Wu et al., 2002; Jang et al., 2007). "Visceral behavioral" studies, using flat sheet preparations of live rodent colon, confirmed the direct role of entero-endocrine gustation in controlling gut motility (Kendig et al., 2014). Monosodium glutamate (MSG), a stimulant of the T1R1/T1R3 umami receptor, when applied to the colon preparations, elicited a peristaltic reflex, which was abolished in preparations from mice that were mutant for T1R1. Interestingly, aside from enteroendocrine cells, another specialized type of vertebrate intestinal cell called "tuft cell" (also "brush cell") expresses gustatory receptors. Tuft cells, found in the respiratory as well as intestinal epithelium, are characterized microscopically by apical "tufts" of elongated microvilli. According to recent studies, they play an important role in the immune response, for which the gustatory signaling cascade may be required. Structurally and developmentally, brush cells differ from enteroendocrine cells (Bjerknes et al., 2012; Gerbe and Jay, 2016), and will not further be considered here. Gustatory receptors are also expressed in the *Drosophila* intestine. Out of the 67 gustatory receptor encoding genes, 15 were expressed in subsets of entero-endocrine cells (Park and Kwon, 2011). They included sugar receptors, but more prominently bitter receptors. A subset of cells expressing tachykinin and diuretic hormone 31 (see below) is stimulated by amino acids in the gut lumen (Park et al., 2016); a direct stimulus-sensing role of the taste receptors remains to be shown experimentally.

Entero-endocrine cells fall into numerous classes, based on the neuropeptide expressed most prominently. More than 15 peptides were identified thus far in the mammalian gut. Recent studies make it abundantly clear that most, if not all of these cell types express combinations of numerous peptides, and that these combinations vary with location along the anterior-posterior axis of the gut. For example, regarding the spectrum of peptides expressed, L-cells (classically defined by their expression of the glucagon-like peptides; GLP1/2) of the proximal intestine resembled more closely the proximal K-cells (defined by expression of glucagon-inhibited peptide; GIP) than the L-cells of the distal intestine (Habib et al., 2012). The same maybe true for the insect gut. In *Drosophila*, 10 different peptides have been described thus far (Veenstra et al., 2008; Veenstra, 2009; Veenstra and Ida, 2014) and are listed in Fig.2, alongside with their most likely vertebrate homologs. The pairing between *Drosophila* and vertebrate peptides is based on several recent systematic studies (Jekely, 2013; Mirabeau and Joly; 2013); in the latter, whole genomes of many different species were used to re-evaluate the relationship between protostome and deuterostome peptidergic systems. Fig.2 also summarizes important aspects of the expression pattern and function of the neuropeptides listed. A number of conclusions can be drawn by comparing the expression and function of these factors:

1. Many insect gut peptides have vertebrate homologs with related function. Insect NPF and vertebrate NPY are involved in controlling food intake (appetite; Brown et al., 1999; Rohwedder et al., 2015; Mercer et al., 2011); DRH31 and CGRPs play a role in controlling gut motility and fluid balance/vascular tone (Sternini, 1991; Coast et al., 2001; Johnson et al., 2005; LaJeunesse et al., 2010; Kendig et

al., 2014); allatostatin C and somatostatin effect gut motility and control developmental growth (Kaminski et al., 2002; Veenstra, 2009a; Corleto, 2010).

2. Many insect gut peptides and their vertebrate homologs are expressed not only in entero-endocrine cells, but also other "nodes" of the brain-gut axis. These nodes include the enteric nervous system, which is derived from the neuroectoderm (neural crest; in vertebrates) or the foregut ectoderm (in insects; Fig.2A1), as well as the brain, derived from the neurectoderm in all animals. Within the brain, specialized centers, such as the hypothalamus in vertebrates, or the pars intercerebralis in insects, are neuroendocrine centers where neurons expressing brain gut peptides are concentrated (Fig.2A2).

3. Whereas some gut peptides are expressed in same nodes of the brain-gut axis (e.g., tachykinin/substance P, expressed in all nodes; Schoofs et al., 1993; Holzer and Holzer-Petsche, 1997; Nässel et al., 1998; Siviter et al., 2000; Hökfelt et al., 2001; allatostatin C/somatostatin, expressed in entero-endocrine cells and brain; Kaminski et al., 2002; Veenstra, 2009a; Corleto, 2010), others have been described for different nodes, even though they maybe involved in the same overall function. For example, DRH31 is expressed in entero-endocrine cells in insects, while its vertebrate counterpart (CGRP) is found in enteric neurons. CCHamide1/2 are also present in gut cells and in the brain (Veenstra and Ida, 2014; Ren et al., 2015; Sano et al., 2015); expression of vertebrate endothelins was not described for endocrine cells or neurons, but for endothelial cells, as well as other epithelia, including the intestinal epithelium (Takizawa et al., 2005). Orexin is a vertebrate peptide expressed in all nodes of the brain gut axis (Heinonen et al., 2008); its insect counterpart, allatotropin, is found in the brain and stomatogastric nervous system, but not in the gut (Bhatt and Horodyski, 1999; Duve et al., 2000; Rankin et al. 2005). It is of course possible that future studies will uncover additional expression patterns for these factors.

Development of the entero-endocrine system

The origin and specification of the entero-endocrine system shows many common features in vertebrates and invertebrates. In both, the gene networks active in entero-endocrine cell specification overlap largely with those controlling the development of neurons. In other words, we find a "brain-gut axis" not only in regard to function, but also to development (Hartenstein et al., 2010). Entero-endocrine cells along with all other intestinal epithelial cells (enterocytes) are of endodermal origin. In the vertebrate embryo, the first entero-endocrine appear at an early stage when the gut forms a simply epithelial tube in which all cells are still mitotically active (Henning et al., 1994; Crosnier et al., 2005; Fig.3A1/A2). Subsequently, the gut epithelium is folded into villi and crypts containing mostly differentiated, postmitotic cells. Proliferation becomes restricted to cells of the crypts, where one finds populations of slowly cycling, self-renewing stem cells; these cells give rise rapidly dividing amplifying progenitors. Cells leaving the mitotic cycle move upwards and differentiate as enterocytes, endocrine cells, and gland cells (Fig.3A3).

The different cell fates are controlled by the expression of bHLH and other transcription factors, whose balanced allocation to different cell populations depends on signaling pathways, notably the Notch pathway. This pathway controls an early developmental choice between continued proliferation (high N activity) and differentiation as a secretory (gland/endocrine) cell (low N activity; Fig.3A3; May and Kaestner, 2010; Schonhoff et al., 2004a). As a result of low N activity levels, the bHLH transcription factor Math1 (=Atoh1, in mammals) or Ascl1 (zebrafish) is expressed in progenitors destined to become secretory cells (Yang et al., 2001; Li et al., 2011; Roach et al., 2013; Flasse et al., 2013; Fig.3A3). This lineage splits up further when (as a result of so far unknown signaling interactions) additional cell fate determinants become active. Expression of the bHLH factor Ngn 3 specifies the fate of entero-endocrine cells (Jenny et al., 2002; Lee et al., 2002; Lee and Kaestner, 2004; Schonhoff et al., 2004a; Fig.3A). Other transcription factors, among them NeuroD1 (Naya et al., 1997; Pax4 and Pax6 (Larsson et al., 1998), Arx (Beucher et al., (2012), Nkx2.2 (Desai et al., 2008), Lmx1a (Gross et al., 2016), and Foxa1/2 (Ye and Kaestner, 2009) are expressed in specific entero-endocrine sublineages.

Drosophila produces two complete intestinal tracts during its life cycle. The larval gut arises from the embryonic endoderm (Fig.3B1/B2). Endocrine cells split from the enterocyte lineage, as well as from progenitors of the adult gut (Takashima et al., 2011a; Fig 3B2). In the larva, adult gut progenitors proliferate (Fig.3B2). In the pupa, these cells generate a new enterocyte epithelium that becomes the adult midgut (Takashima et al., 2011b, c; 2016; Fig. 3B3). In addition, a subset of adult gut progenitors is set aside as presumptive intestinal stem cells, which divide throughout the pupal and adult phase, and supply the adult endocrine cell population, as well as renewed stem cells that replace all intestinal cell types (Takashima et al., 2016; Fig.3B4). The fate of the endocrine lineage is dependent on the expression of the bHLH gene *scute* (*sc*) and *asense* (*ase*), homologs of vertebrate *ascl1* (Amcheslavsky et al., 2014; Wang et al., 2015), as well as other proneural genes (see below). As in vertebrates, these determinants are upregulated, first in subsets of endoderm cells and later in subsets of intestinal stem cells, as a result of decreased N activity, and specifies them as entero-endocrine precursors. The *Drosophila* midgut lacks specialized excretory gland cells, except for acid producing cells that are restricted to a narrow segment in the midportion of the gut (Shanbhag and Tripathi, 2009; Marianes and Spradling, 2013). Interestingly, these cells, like enterocytes, but opposed to endocrine cells, require high N activity (Strand and Micchelli, 2011).

Many aspects of *Drosophila* entero-endocrine cell specification and morphogenesis have remained elusive. One question is whether the specific type of cell, in terms of the peptide(s) it releases, is determined at the level of the stem cell. In other words, does a given stem cell give rise to only one type of entero-endocrine cell, or a mixed population? How does an immature entero-endocrine cell, which starts out as an unpolarized, mesenchymal precursor, become polarized and insert itself in the intestinal epithelium? Is the *Drosophila* cognate of the bHLH gene Ngn3, which plays a crucial role in determining the entero-endocrine fate in vertebrates, of similar importance in the fly intestine? To approach these questions, we have analyzed the early development of the adult population of entero-endocrine cells during metamorphosis.

Material and Methods

Fly stocks and maintenance

Fly lines used in this study were: *OregonR^w1118*, *UAS-myr-mRFP*, *UAS-mCD8GFP*, *UAS-flp*, (all provided by the Bloomington Stock Center); *esg-Gal4* (the National Institute of Genetics, Mishima, Japan). Flies were maintained with normal fly food at room temperature or in incubators set at 25°C or 18°C. In some experimental conditions, flies were transferred to the temperature at 29°C to suppress the function of *Gal80^ts*. Heat-shock was provided at 37°C variable lengths of time. For mitotic (MARCM) clones (Lee and Luo, 2001) we used progeny with the genotype *hsflp/+; FRTG13, UAS-mCD8GFP/FRTG13, tub-GAL80; tub-Gal4/+*. Clones were induced during the late third instar by heat-shocking at 38°C for 30 minutes to 1 hour. Numerical aspects of clone sizes were analyzed in a previous paper to reconstruct the proliferatory behavior of presumptive intestinal stem cells of the pupal midgut (Takashima et al., 2016). In this work we used double labeling with different antibody markers (see below) to evaluate distribution of different types of peptides in clones.

Immunohistochemistry

Antibody and in situ staining was performed as described previously (Ashburner, 1989; Takashima et al., 2011; 2016). A digoxygenin-labeled RNA probe prepared against *tap* was used. Antibodies used were (dilutions in parentheses): mouse antibodies against Discs large (Dlg, 1:10), Prospero (Pros, 1:50) (all obtained from the Developmental Studies Hybridoma Bank, University of Iowa), rabbit antibodies against FMRF-amide which recognizes the enteric peptide NPF (1:500; Thermo Fisher Scientific, Waltham, MA), Tachykinin (1:2000; a gift from Dr. Dick Nässel), goat secondary antibodies against mouse IgG labeled Alexa488 (1:100), mouse IgG Alexa 546 (1:300), rabbit IgG Alexa488 (1:100) (Life Technologies); rabbit Cy3 (1:200) (Jackson Immunoresearch, West Grove, PA).

Results

Clonal relationships and morphogenesis of Drosophila entero-endocrine cells

To study the relationship between different cells, lineage tracing experiments and labeled clones, generated by markers activated in individual proliferating progenitor cells, provide the most direct approach. In vertebrates, this technique has demonstrated that individual progenitors can produce different types of endocrine cells (e.g., Rindi et al., 1999; Schonhoff et al., 2004b; Bjerknes and Cheng, 2006). To address this question in *Drosophila*, we induced GFP-labeled MARCM clones in individual midgut progenitors at the late larval/early pupal stage, using *esg*-Gal4, a transgene specifically expressed in presumptive intestinal stem cells from the embryonic to the pupal period (Jiang and Edgar, 2009; Takashima et al., 2011; 2016), as a driver line. Midguts were fixed at stages 72h after puparium formation (APF), 96h, and 1–2 days after eclosion of the adult fly. Preparations were labeled with an antibody against Prospero (Pros), a global marker for all entero-endocrine cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), and against Tachykinin, which is expressed in a subset of these cells (Veenstra et al., 2008). For the adult, a subset of specimens were labeled with both antibodies against two different (families of) peptides, anti-Tachykinin and anti-FMRFamide (recognizes NPF).

Pupal pISCs divide in several parasynchronous waves, initially generate lineages composed exclusively of additional pISCs and endocrine cells (Guo and Ohlstein, 2015; Takashima et al., 2016). This is different from the adult, where ISCs produce mainly enterocytes. As reported previously (Takashima et al., 2016), labeled clones fixed around 72 h APF were comprised of 4–5 small, sub-epithelial cells, including Pros-negative pISCs and Pros-positive endocrine cells. The latter amounted to about half of the clone (2.1±0.3 s.d; Fig.4A–C). At 96, clone size had increased to 6.0 (± 1.8 s.d.), shortly after eclosion to 7.8 (+1.1 s.d.; n=68; Fig.4D, E). For both time points, the number of Pros-positive endocrine cells did not increase significantly, supporting the idea that these are postmitotic cells. Importantly, in almost all clones from 72h APF onward, endocrine cells included both Tachykinin-positive and Tachykinin-negative endocrine cells (Fig.4A–E). Among the adult clones labeled with two antibodies against different peptides (NPF and Tachykinin), the majority had a cell that expressed both peptides, and a sibling cell expressing only Tachykinin (Fig.4E). These findings demonstrate that pISCs have the potential to produce endocrine cells with different peptides (Fig.4F).

Morphogenesis of entero-endocrine cells in the pupal intestine

The first Pros-positive entero-endocrine cells appear around 50–60hAPF (Fig.5A). The expression of different peptides, including FMRFamides and Tachykinin, can be detected around 72h APF (Fig.5B). At this stage, entero-endocrine cells are still rounded, unpolarized cells residing at the base of the gut epithelium (Fig.5B, E1). At 96h APF, most entero-endocrine cells have become polarized, extending narrow apical "necks" that establish contact with the lumen of the midgut (Fig.5C, E2). This step is accompanied by the formation of a junctional complex between entero-endocrine cell and adjoining enterocyte. An antibody against Disc-large (Dlg), which forms part of septate junctions (Woods and Bryant, 1991), reveals the apically located junctional complex in the pupal gut epithelium. At 72h APF, all enterocytes are surrounded apically by a robust Dlg-positive junctional complex (Fig.5F-F'''). Subepithelial Pros-positive endocrine precursors are excluded from this apical junction. By 96h APF, many small-diameter rings of anti-Dlg positive material can be detected in the apical layer of the gut epithelium (Fig.5G-G''', arrow). These rings can be interpreted as the junctional complex formed around the narrow apical necks of endocrine cells which, by this stage, have penetrated into the gut epithelium (Fig.5C). Around the time of eclosion and shorty thereafter, further differentiation of endocrine cells take place. For example, microvilli, which form a conspicuous "brush border" in fully differentiated enterocytes and endocrine cells (Fig.5I, E3) are still sparse and short in the late pupal intestine (Fig.5H).

Role of the proneural gene Tap in entero-endocrine development

Proneural bHLH transcription factors play a pivotal role in the specification of entero-endocrine cells in vertebrates. In mammals, Math1, a member of the atonal family of bHLH genes, is expressed in all intestinal progenitors destined to become secretory (including gland and endocrine) cells (Yang et al., 2001). In zebrafish, Ascl1, a achaete-scute-like bHLH factor, appears to be the first gene signaling a secretory fate (Roach et al., 2013; Flasse et al., 2013). Downstream of Math1, a bHLH factor of the neurogenin family, Ngn3, is expressed in a smaller subset of progenitors which embark on the endocrine fate (Jenny et

al., 2002). Lack of these factors result in the loss of endocrine cells; mutation the human ngn3 gene causes the congenital malabsorptive diarrhea syndrome (Wang et al., 2006). In *Drosophila*, the gene *scute* (cognate of vertebrate *ascl1*) is the bHLH gene expressed early and strongly expressed in intestinal endocrine precursors, both in the embryonic endoderm (Fig.6A, B), and later in the pupa (not shown). The same applies to *asense*, another member of the same bHLH family, which is expressed in endocrine precursors after they have split from the endoderm (Fig.6C, D), and, in the pupa, in nascent endocrine cells (data not shown). Loss of *scute* results in the total absence of endocrine cells in the adult gut (Amcheslavsky et al., 2014). Atonal is also found in subsets of endocrine cells, as well as ISCs (S.T. and V.H., unpublished observation).

We were particularly interested to investigate the *Drosophila* counterpart of Neurogenin 3 which, in mammals, defines the entirety of the entero-endocrine lineage. The neurogenin family of bHLH genes is represented by one *Drosophila* cognate, named *target of pox-neuro* (*tap*; Bush et al., 1996; 1999; Gautier et al., 1997). As the name implies, *tap* was identified as a target of the pax gene *pox-neuro* in a subset of *Drosophila* chemosensory neurons (Gautier et al., 1997). Loss of *tap* function results in defined sensory deficits (Ledent et al., 1998), suggesting that the sensory neurons normally expressing *tap* are missing or deficient in the absence of this gene. Using a cDNA probe against *tap* and a *tap*-Gal4 line (Jenett et al., 2012) we discovered that the gene is also expressed in precursors of endocrine cells in the embryo, pupa, and the adult midgut (Fig.6E–H''). Expression is seen only once these cells become postmitotic, from stage 13 onward. The level of expression is not uniform in all endocrine cells, but appears highest in the anterior-to-middle section of the midgut primordium (Fig.6F). In the pupa, *tap*-Gal4>mcd8-GFP expression appears at late stages (>72h APF) in immature endocrine cells (not shown). A similar expression is seen in the adult midgut, where most *tap*-positive endocrine cells, some of them expressing tachykinin, had a rounded shape and subepithelial position (arrowheads in Fig.6H', H''); differentiated, flask-shaped endocrine cells were devoid of *tap* signal (arrows in Fig.6H', H'').

To address the function of *tap* in entero-endocrine cell development, we used a UAS-*tap*RNAi construct driven by *esg*-Gal4. RNAi knockdown from late larval stages onward resulted in midguts in which both endocrine cells and ISCs were strongly reduced (Fig.6K, K'). In controls (Fig.6J, J'), the ratio between these two cell types (whose number stays constant between onset of metamorphosis and eclosion; Takashima et al., 2016) is 10.6% (s.d.=1.1); the ratio between ISCs and enterocytes is 19.2% (s.d.=1.4). In the L3-Adult *tap* knockdown, the endocrine/enterocyte ratio was reduced to 3.3% (s.d.=3.0), the ISC/enterocyte ratio to 2.4% (s.d.=0.9). This finding suggests that *tap*, expressed in endocrine cells, plays a role in maintaining ISCs and endocrine cells alike. Further support for this conclusion comes from temporally controlled *tap*-RNAi expression right after adult eclosion, and analyzing the shape and distribution of ISCs at increasing time intervals after knocking down *tap* (Fig.6L–N'). The most dramatic effect can be seen in ISCs, which decrease in size and number, and lose their horizontal processes, which normally spread out over the basal surface of the intestinal epithelium (Fig.6M, N). Seven days after onset of the *tap*-knockdown, the ISC/enterocyte ratio was reduced to 4.8% (s.d.=2.7). Our results indicate that a feedback signal between endocrine cells (expressing *tap*) and the stem cells that produced them is involved in stem cell maintenance and endocrine cell production (Fig.6I).

Discussion

Cell lineage and entero-endocrine identity in the Drosophila pupal gut

Our results demonstrate that endocrine cells expressing different gut peptides derive from individual pISCs, rather than forming separate lineages. A large fraction of clones analyzed had only two endocrine cells which differed in their peptide expression pattern, indicating suggesting that the "choice" of peptide is made in the postmitotic endocrine cell. These findings confirm the results of a recent study by Beehler-Evans and Micchelli (2015) on the adult posterior fly midgut, where individual, labeled ISCs also gave rise sets of endocrine cells expressing different combinations of peptides.

The independence of peptide expression from lineage might also be true for vertebrates. Here, numerous bHLH and other transcription factors are expressed in specific combinations that can be assigned to specific types of endocrine cells (reviewed in Moran et al., 2008; Brubaker, 2012; Latorre et al., 2016). One possible model might assume that the expression pattern of these fate determining factors is fixed at the level of the dividing progenitors: one type of progenitor might express the combination A and B, the other B and C; proliferation of the A/B-expressing progenitor would generate one type of endocrine cell, and the B/C expressing one another type. In the other extreme, all progenitors might be equivalent, and the decision to express A/B vs B/C is made postmitotically. Clonal analyses and lineage tracing studies favor a model that is closer to the second rather than the first extreme. For example, Ngn3-expressing cells are mostly postmitotic endocrine cells; only about 30% are dividing progenitors (Bjerknes and Cheng, 2006). Other studies showed that the proliferating progenitors that start to express a certain peptide are still not committed to produce only endocrine cells of that type. For example, when inserting the gene herpes simplex virus 1 thymidine kinase in secretin-expressing progenitors, which makes these cells susceptible to the drug ganciclovir, not only were the secretin-positive cells affected, but also other endocrine cell types (CKK, peptide Y, GLP-1; Rindi et al., 1999). This result demonstrates that secretin-expressing progenitors produce progeny that switch to the expression of other peptides.

Origin and morphogenesis of entero-endocrine cells

We have emphasized in this paper the parallels that exist between sensory neurons in terms of function and development. Developmental parallels are particularly pronounced when considering the morphogenetic process that gives rise to the two cell types. Sensory neurons, along with non-neuronal support cells, form part of small lineages derived from subepidermal sensory organ progenitors (SOPs), similar to the basally located pISCs and ISCs of the midgut. SOPs divide asymmetrically, resulting in the delivery of the neuronal determinant, Prospero, to one of the daughter cells, which becomes specified as the sensory neuron (Manning and Doe, 1999; for recent review, Furman and Bukharina, 2012). In pISCs, expression of Pros is turned on in a subset of pISCs which then acquire the fate of entero-endocrine cells (Takashima et al., 2011; 2016; Guo and Ohlstein, 2015).

The following morphogenetic step by which sensory neurons and endocrine cells move into the epithelium also bears a resemblance that is worthy to point out. Both types of cells are

born at a subepithelial position. Subsequently, they extend an apical process that pushes into the epithelium, the epidermis in case of sensory neurons (Hartenstein, 1988), and the intestinal epithelium in case of the enteroendocrine cells (this work). When the process reaches the apical surface, a junctional complex is established with neighboring epithelial cells, and apical specializations (e.g., sensory cilium in case of the neuron, microvilli in the endocrine cell) are formed. Sensory neurons and entero-endocrine cells form two of the rare examples where polarity and epithelial characteristics are acquired de novo in a cell that arises outside the epithelium; they may provide useful paradigms to analyze the process by which epithelial cells acquire and maintain polarity.

Tap, a member of the proneural gene cascade specifying entero-endocrine cells in Drosophila

Proneural genes of the bHLH superfamily of transcription factors are expressed in subpopulations of neurectodermal cells (proneural clusters) on whom they convey the potential to give rise to neural cells. At least five different subfamilies of bHLH genes, all of them containing proneural genes, are thought to have existed at the level of the bilaterian ancestor (Simionato et al., 2008). They include the *achaete-scute, atonal, neurogenin, olig*, and *NeuroD* groups of genes (the latter two thought to be lost in *Drosophila*; Simionato et al., 2008). Once proneural genes have been turned on in a proneural cluster, they set in motion a signaling mechanism, mediated by the Notch signaling pathway, that restricts their own expression to a subset of cells within the proneural cluster (lateral inhibition). In very general terms, the relationship between different proneural genes during neural development can be described in two different ways. On the one hand side, different proneural genes are expressed and required at different neurectodermal locations, for different types of neurons. For example, *Drosophila atonal* plays a role in the formation of chordotonal organs (stretch receptors), photoreceptors, some olfactory receptors, and a single neural lineage in the brain (Jarman et al., 1993a; 1994; Gupta and Rodrigues, 1997; Hassan et al., 2000); other parts of the nervous system do not express or require the gene. A similarly restricted function can be attributed to the mouse *atonal* homologs (e.g., *Atoh1, Atoh5*; reviewed in Hsiung and Moses, 2002; Quan and Hassan, 2005; Mulvaney and Dabdoub, 2012). Secondly, different proneural genes may act sequentially in the same proneural cluster in a cascade, whereby an early expressed proneural gene directly promotes the transcription of a later one. In this manner, the genes *l'sc* or *sc*, expressed in the *Drosophila* neurectoderm prior to neuroblast delamination, activate *ase*, which appears once neuroblasts have segrated and divide (Brand et al., 1993; Jarman et al., 1993b; Quan and Hassan, 2005).

The role of proneural genes in entero-endocrine development appears to be very similar to that in the nervous system. Not only do we encounter the same players (proneural genes = proendocrine genes), but also interaction between genes (e.g., Notch–mediated lateral inhibition; sequential activation of different proneural/proendocrine genes) are conserved. This has been well studied for the mammalian "proendocrine" cascade of genes, where, first, Atoh1 expression defines all secretory lineages among the intestinal progenitors; this is followed by the expression of Ngn3 and NeuroD1, which act downstream of Atoh1 in the endocrine lineage (reviewed in Li et al., 2011). It is clear that, in addition to this temporal restriction, genes like Ngn3 or NeuroD1 are also spatially/cell type restricted; for example,

many entero-endocrine cells of the stomach do not express or require Ngn3 (Jenny et al., 2002).

The expression and interaction of proneural/proendocrine genes differ significantly when comparing different animal clades. In zebrafish, *atoh1* does not appear to play a role at an early stage of intestinal development to specify secretory lineages. Instead, the achaete-scute family member, *ascl1*, carries out this function (Roach et al., 2013; Flasse et al., 2013). Ngn3 is expressed in subsets of entero-endocrine cells in the zebrafish gut, at later stages than *ascl1*, but does not to have a similarly widespread role as in mammals (K.Wallace, pers comm). In *Drosophila*, it also appears to be an *achaete-scute* bHLH gene, *sc*, which acts as the earliest and most widespread determinant for the entero-endocrine lineage (Amcheslavsky et al., 2014). The atonal bHLH member, *ato*, appears to have a more restricted expression (V.H. and S.T., unpublished observation), but an in depth analysis of the function of this gene in the fly intestine has not yet been carried out.

The results reported here indicate that the one *Drosophila* neurogenin-type bHLH family member, *tap*, also plays a role for the commitment and differentiation of the entero-endocrine lineage. *Tap* is expressed transiently in entero-endocrine cells in the embryo, pupa and adult, and appears to be downregulated when these cells terminally differentiate. As in vertebrates, *tap* expression starts at a later stage than that of the "primary" endocrine determinant, *sc*. Knockdown of *tap* in pupal pISCs results in a loss of endocrine cells, but also of the pISCs themselves; a similar phenotype is seen when knocking down *tap* in adult ISCs. These findings, which need to be supplemented by additional studies using different types of Gal4 driver lines to express *tap*RNAi, as well as clonal analyses removing the *tap* gene in individual progenitors, nevertheless point at a role of *tap* for endocrine cell maintenance/differentiation. Specifically, our findings support the results of recent works that established signaling interactions between endocrine cells and ISCs which were both required to inhibit the production of further endocrine cells (lateral inhibition), as well as to maintain ISCs (Fig.6I). Endocrine cells emit the signal Slit that acts on Robo2, expressed by ISCs, and tilts the "decision" of these cells away from generating additional endocrine cells (Biteau and Jasper, 2014).

Acknowledgments

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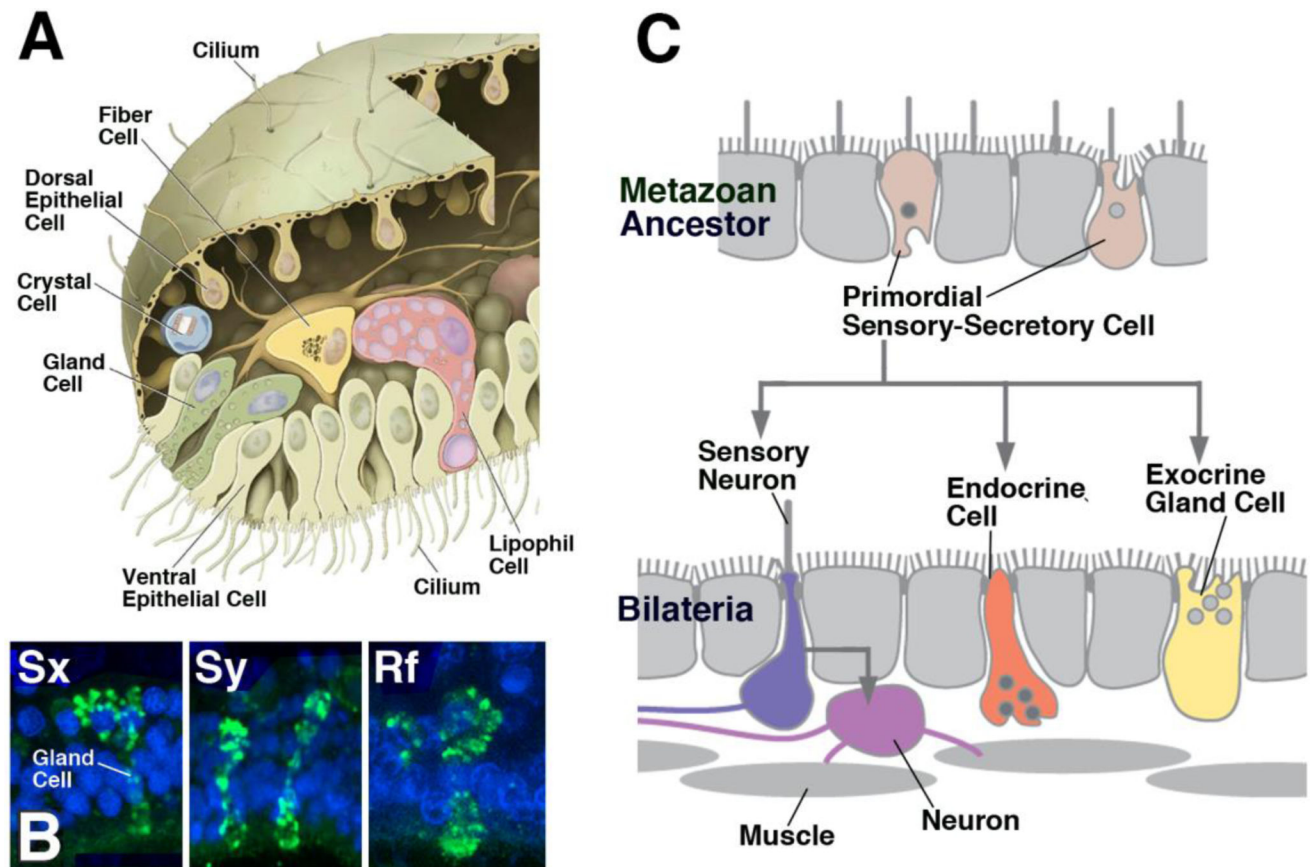
Figure 1.
(A) Cut away diagram of placozoan, showing ciliated epithelial cells, gland cells and other cell types (from Smith et al., 2014, with permission). (B) Immunohistochmical detection of synaptic proteins [syntaxin (sx); synapsin (sy)] and peptide FMRFamid (Rf) in placozoan gland cells (from Smith et al., 2014, with permission). (C) Schematic depicting hypothetical evolutionary origin of bilaterian neurons and enterocendocrine. The shared characteristics between these cell types can be interpreted as "deep homologies", inherited from a hypothetical type of sensory-secretory cell already present in basal metazoan.
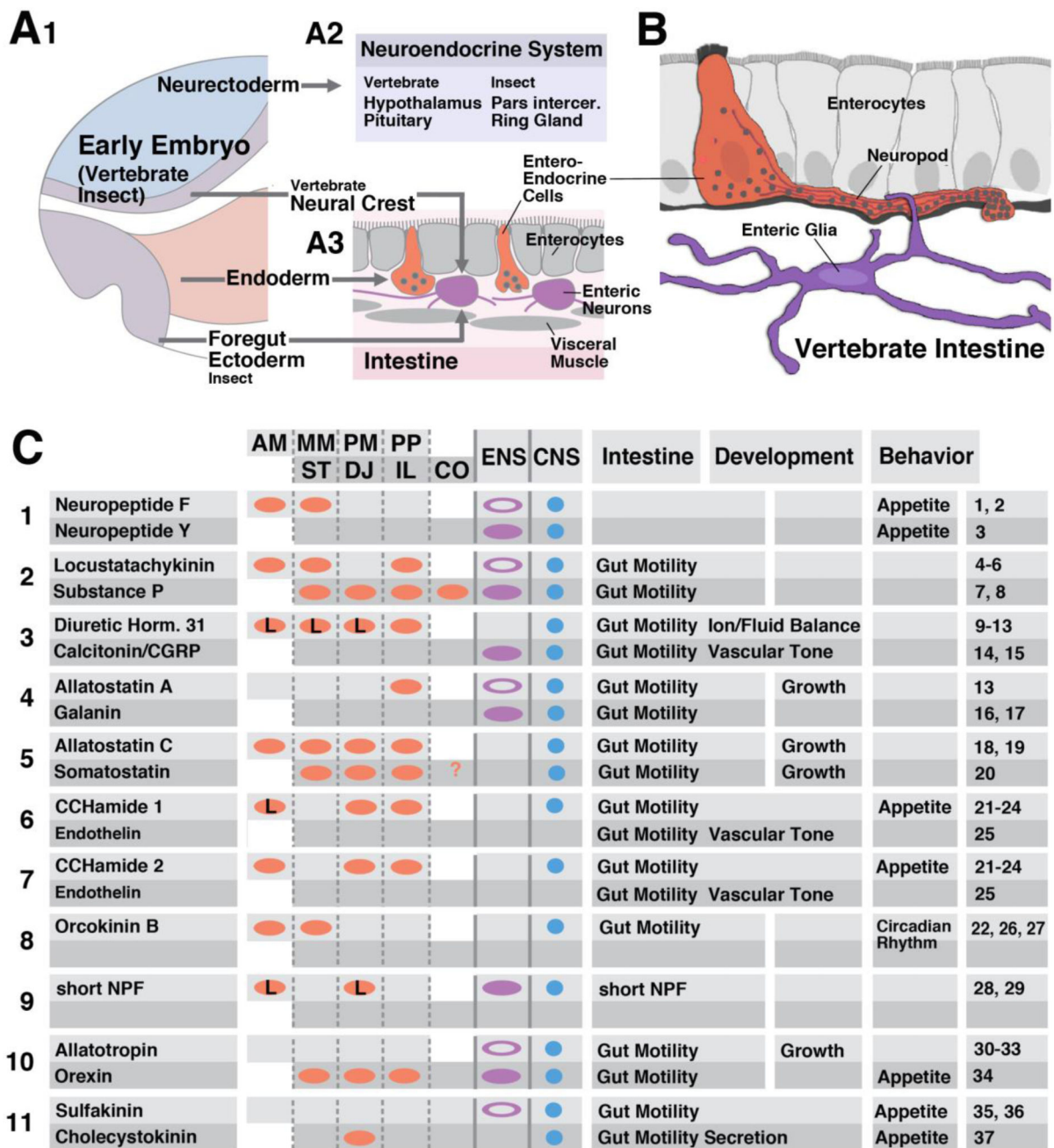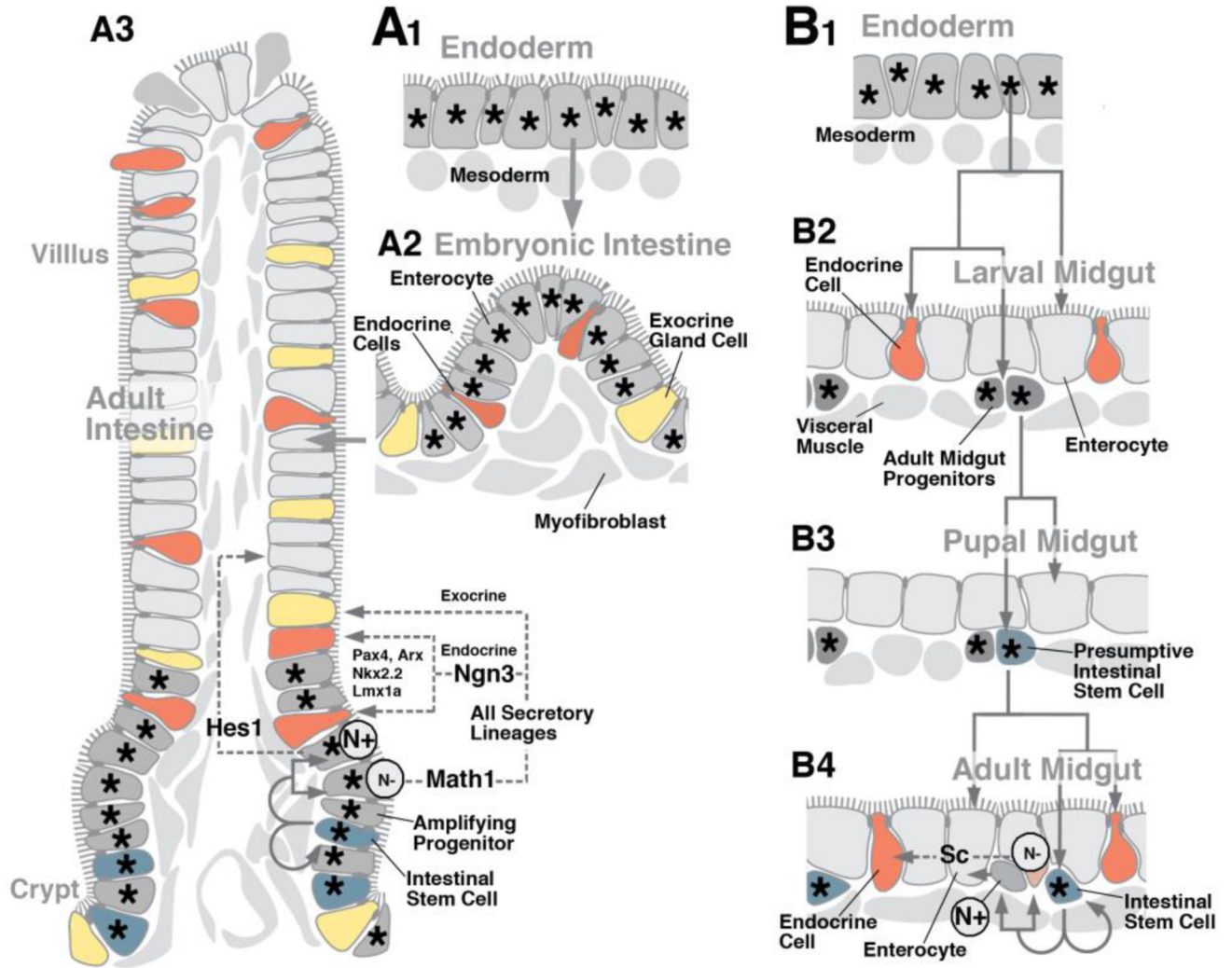
Figure 2.
(A) Ontogeny of the brain-gut axis, including entero-endocrine cells (from endoderm), enteric neurons (migrating from neural crest in vertebrates and foregut ectoderm in arthropods), and neurosecretory (neuroendocrine) cells. (A1) shows schematic view of generalized bilaterian embryonic head; (A2) represents neuroendocrine system associated with brain; (A3) presents schematic section of midgut. (B) Reconstruction of entero-endocrine cell in mouse small intestine, extending basal process ("neuropod") into enteric plexus where it interacts with glial processes (from Bohorquez et al., 2014, with permission). (C) Comparison of expression and function of selected brain-gut peptides.

Upper half (light gray) of rows 1–9 list the peptides identified in *Drosophila* entero-endocrine cells (Veenstra et al., 2008; Veenstra, 2009; Veenstra and Ida, 2014). Putative vertebrate counterparts (based on Mirabeau and Joly, 2013; Jekely, 2013) are shown below (dark gray shading). Columns in center of table schematically depict expression of peptides [red: expression in entero-endocrine cells; purple: enteric nervous system (ENS; oval is closed when expression is verified in *Drosophila*; oval is open when expression has been reported only for insect species other than *Drosophila*); blue: central nervous system (CNS)]. Abbreviations for segments of vertebrate intestine: ST stomach; DJ duodenum and jejunum; IL ileum; CO colon. Abbreviations for parts of *Drosophila* intestine: AM anterior midgut; MM middle midgut; PM posterior midgut; PP terminal segment of posterior midgut; L peptide present only in larval gut. The alignment of segments of vertebrate and insect gut is based on the following considerations. (1) The middle segment of the insect midgut (MM), which contains acid-producing gland cells, is aligned with the stomach (ST) of the vertebrate gut. (2) The colon (CO) of the vertebrate gut, which contains endocrine cells, is considered developmentally as "hindgut". As such, it does not have a counterpart in insects, where the "hindgut" denominates the ectodermally derived, endocrine cell-lacking part of the intestinal tract. (3) Similarly, the anterior midgut of insects (AM) contains endocrine cells, which finds no correspondence in vertebrates (the esophagus, according to our current knowledge, does not possess endocrine cells). Columns at the right highlight experimentally confirmed effects of peptides in intestinal function, during development, and feeding behavior, based on references indicated by numbers. Rows 10 and 12 show two other intestinal peptides from vertebrate, orexin and cholecystokinin, with insect counterparts expressed in enteric and central neurons.

References for expression and function of peptides:

(1) Brown et al., 1999; (2) Rohwedder et al., 2015; (3) Mercer et al., 2011; (4) Schoofs et al., 1993; (5) Siviter et al., 2000; (6) Nässel et al., 1998; (7) Holzer and Holzer-Petsche, 1997; (8) Hökfelt et al., 2001; (9) Coast et al., 2001; (10) Johnson et al., 2005; (11) Brugge et al., 2008; (12) LaJeunesse et al., 2010; (13) Vanderveken and O'Donnell, 2014; (14) Sternini, 1991; (15) Kendig et al., 2014; (16) Rattan and Tamura, 1998; (17) Anselmi et al., 2005; (18) Kaminski et al., 2002; (19) Veenstra, 2009; (20) Corleto, 2010; (21) Farhan et al., 2013; (22) Veenstra and Ida, 2014; (23) Ren et al., 2015; (24) Sano et al., 2015; (25) Takizawa et al., 2005; (26) Sterkel et al., 2012; (27) Chen et al., 2015; (28) Carlsson et al., 2013; (29) Slade and Staveley, 2016; (30) Bhatt and Horodyski, 1999; (31) Duve et al., 2000; (32) Rankin et al., 2005; (33) Audsley et al., 2008; (34) Heinonen et al., 2008; (35) Duve et al., 1994; (36) Audsley and Weaver, 2009; (37) Dockray, 2009.

Figure 3.
Schematic depicting development of entero-endocrine system in vertebrate (A) and *Drosophila* (B). Panels illustrate developmental stages, from developing endoderm (embryo: A1/A2; *Drosophila* larva: B2; *Drosophila* pupa: B3) to adult gut (A3, B4), with enterocytes rendered gray, endocrine cells red, gland cells yellow. Dividing progenitors are indicated by asterisk; adult stem cells are rendered blue. Conserved genetic mechanisms specifying entero-endocrine cells are indicated at bottom of panels. Cell-cell interactions among progenitors, mediated by the Notch pathway, distinguish between enterocyte fate (high levels of Notch activity, indicated by N+) and secretory/entero-endocrine fate (low Notch activity indicated by N−). bHLH transcription factors, upregulated as a result of decreased N activity, specify endocrine cells (see text for details).
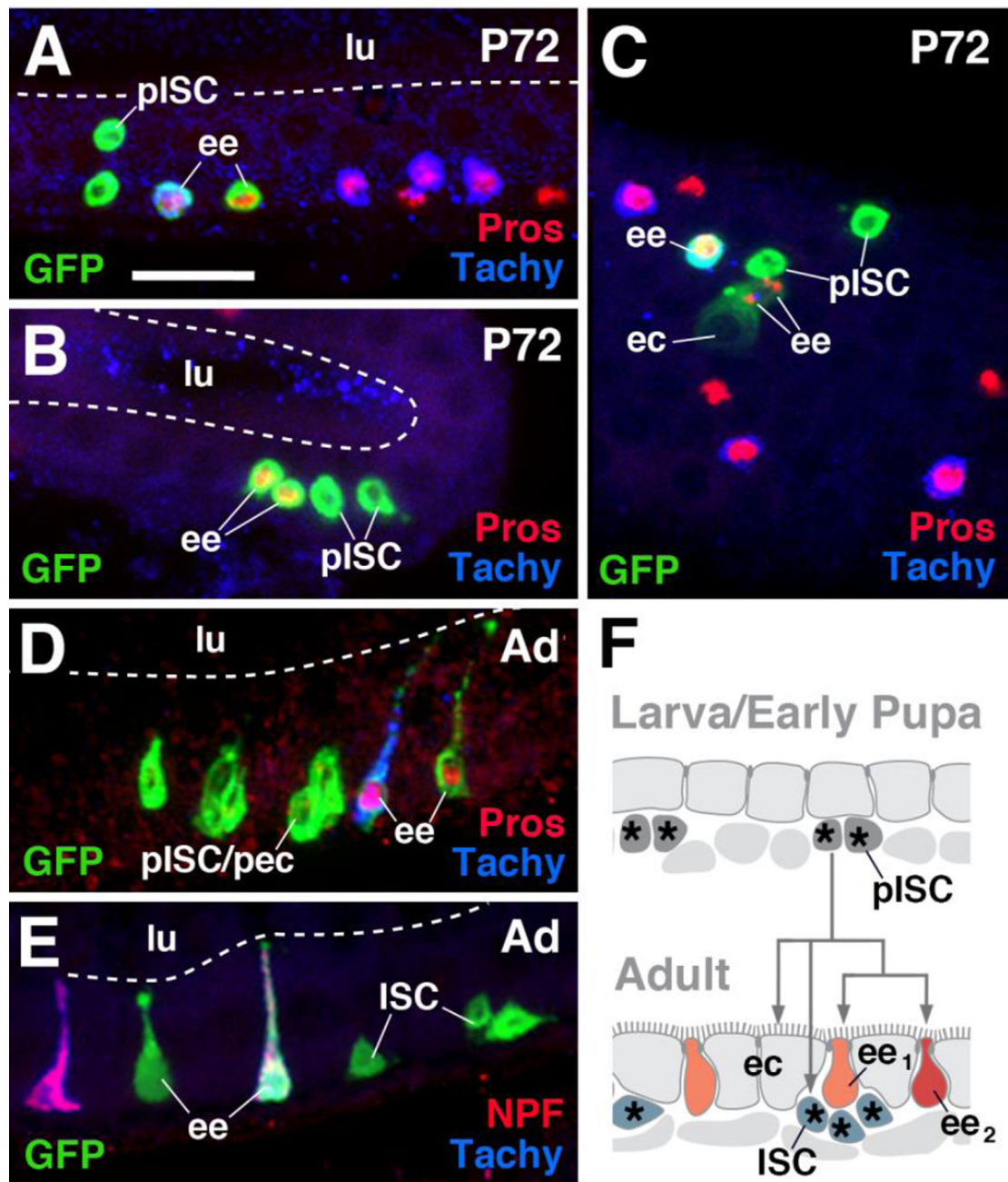
Figure 4.
Clonal analysis of *Drosophila* entero-endocrine cells. Panels (A)–(E) are z-projections of confocal sections of midgut epithelium. MARCM clones (GFP; green) were induced in late larval/early pupal pISC and fixed at 72hrs after puparium formation (P72; A–C) and shortly after eclosion (Ad; D, E). Entero-endocrine cells were marked by expression of Prospero (Pros; red). Clone shown in (C) was induced in midgut progenitor of late larva, prior to the split between pISC and enterocyte; as a result, pupa enterocyte (ec) is included in the clone. At P72, entero-endocrine progeny (ee) included Tachykinin-positive cells (blue) as well as Tachyinin-negative progeny; in adult (D, E), two different peptides (Tachykinin, blue; NPF,
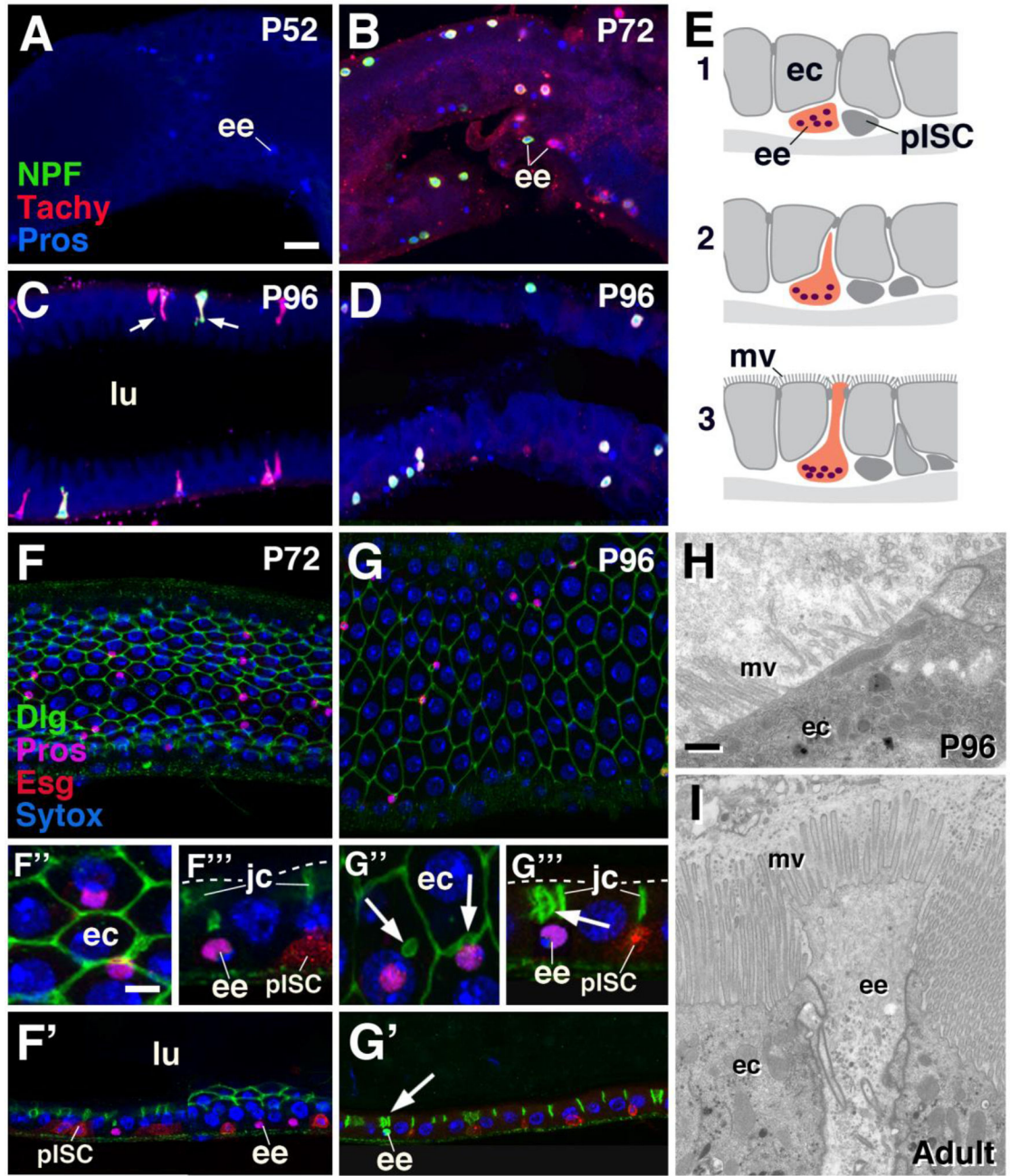
red) could be detected within entero-endocrine cells of a single clone. (F) Schematic summary of clonal analysis. Hatched line in A–E indicates apical surface of midgut epithelium. Other abbreviations: lu lumen of midgut; pec presumptive enterocyte. Bar: 10μm
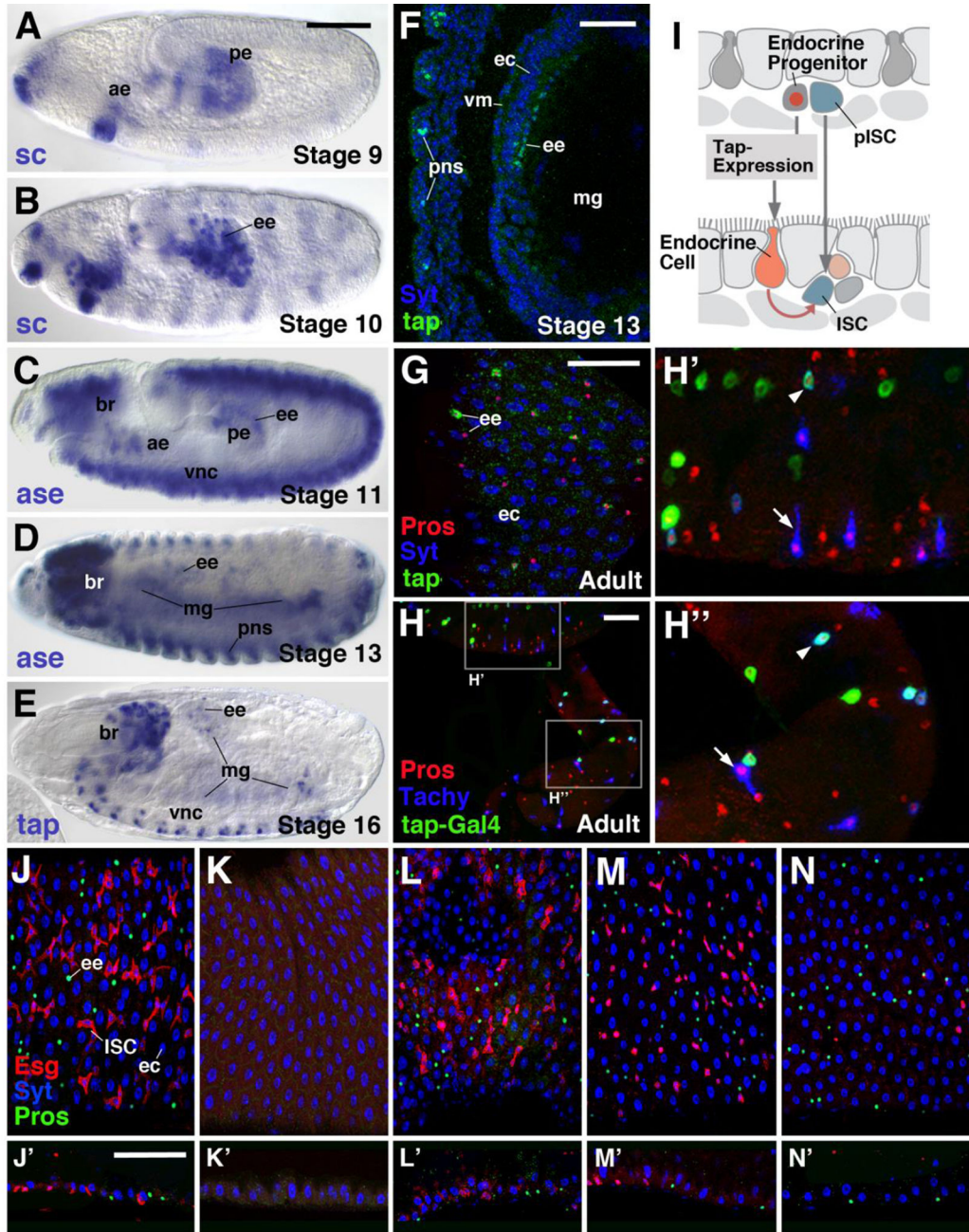
Figure 5.
Morphogenesis of entero-endocrine cells during pupal development. Panels (A–D) and (F–G''') are z-projections of confocal sections of midgut at 52 hrs after puparium formation (P52; A), 72 hrs (P72; B, F-F'''), and 96 hrs (P96; C, D, G-G'''). (F and F''), and (G and G'') show tangential sections (plane of the epithelium); (F' and F''') and (G' and G''') are cross sections of epithelium with apical membrane facing up. (H, I) are transmission electron microscopy sections of apical part of midgut epithelium of late pupa (P96, H) and adult (I). (E) schematically summarizes steps in morphogenesis of entero-endocrine cells. Pros-positive entero-endocrine precursors appear around P52, but do not yet express peptides at

immunohistochemically detectable levels (A). At P72 (B), entero-endocrine precursors start expressing peptides (NPF: green; Tachykinin: red). They are rounded, subepithelial cells with no apical junctional complex and contact to midgut lumen (lu; F-F'''). One day later, most entero-endocrine cells have extended slender apical processes into the anterior and posterior intestinal epithelium (arrow in C); most cells in the middle part of the midgut still lack apical processes (D). A junctional complex surrounding the apex of the processes [labeled by the septate junction marker anti-Discs large (green)] forms conspicuous ring-shaped or barrel-shaped figures (arrows in G'-G'''). Pros-positive nuclei of ennteroendocrine cells in (F–G''') are manually rendered magenta to set them apart from Dlg-positive junctional complex (green). Apical membrane specializations, in particular the dense brush border formed by long, strictly parallel microvilli (mv) in enterocytes (ec) and endocrine cells (ee) of the adult midgut (I), are still immature in pupal midguts shortly before eclosion [P96 in panel (H)]. Bars: 10μm (A–D; F, F', G, G'); 3μm (F", F''', G", G'''); 0.5μm (I, H)

Figure 6.

(A–F) Wholemount in situ hybridization showing expression of the bHLH genes *scute* (*sc*; A, B) *asense* (*ase*; C, D) and *target-of-pox-neuro* (*tap*; E, F) in embryonic endoderm (ae anterior endoderm; pe posterior endoderm; A–C) and midgut primordium (mg; D, E, F). (A–E) show lateral views, anterior to the left (courtesy of Berkeley Drosophila Genome Project data base; http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl). (F; fluorescent in situ probe in green) presents dorsal view of left side of embryo (anterior to the top), showing *tap* expression in endocrine precursors (ee) and sensory neuronal precursors (pns). (G) Fluorescent wholemount in situ hybridization showing expression of *tap* (green) in subset of endocrine

cells (ee; labeled by anti-Prospero in red) in adult posterior midgut. (H-H") Expression of *tap* reporter (*tap*-Gal4; green) in subset of endocrine cells in adult midgut. Anti-Prospero (red) labels all endocrine cell nuclei; anti-Tachykinin (blue) labels subset of endocrine cells. Note that most *tap*-Gal4-positive cells (representative cell shown by arrowhead in H' and H") lack the apical process, the hallmark of fully differentiated endocrine cells (arrows in H' and H"). (I) Schematic diagram showing developing midgut (top) where progenitors of endocrine cells express tap (red), which is required for differentiation to mature endocrine cells (bottom). (J–N') Confocal section of adult posterior midgut (J–N: tangential section in plane of epithelium; J'–N': cross section) in which intestinal stem cells (ISCs) are labeled by *esg*-Gal>UAS-RFP (red) and endocrine cell nuclei by anti-Prospero (green); nuclei of enterocytes (ec) and all other cells also labeled by Sytox (Syt; blue). (J, J') Control. (K, K') Midgut 5d post eclosion after expressing UAS-*tap*RNAi, driven by *esg*-Gal4 from late larval to adult. Note absence of endocrine cells and ISCs. (L, L') Midgut 1d post eclosion in which tapRNAi was activated at eclosion. (M, M') Midgut 3d post eclosion in which tapRNAi was activated at eclosion. (N, N') Midgut 7d post eclosion in which *tap*RNAi was activated at eclosion. Note progressive rounding up and loss of pISCs in gut where *tap*RNAi was activated.

Other abbreviations: br brain; ec enterocyte; ISC intestinal stem cell; pISC presumptive intestinal stem cell; vm visceral muscle; vnc ventral nerve cord; Bars: 50μm (A–E; G, H; J–N'); 20μm (F)