1 **Models and Tools for Studying Enteroendocrine Cells**

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22

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24 **Abstract**

25 Gut hormones produced by gastrointestinal enteroendocrine cells modulate key
26 physiological processes including glucose homeostasis and food intake, making them
27 potential therapeutic candidates to treat obesity and diabetes. Understanding the function
28 of enteroendocrine cells and the molecular mechanisms driving hormone production is a
29 key step towards mobilising endogenous hormone reserves in the gut as a new therapeutic
30 strategy. In this review we will discuss the variety of ex vivo and in vitro model systems
31 driving this research and their contributions to our current understanding of nutrient
32 sensing mechanisms in enteroendocrine cells.

33

34

35 **Abbreviations**

36 5-HT, serotonin
37 CASR, calcium-sensing receptor
38 CCK, cholecystokinin
39 EEC, enteroendocrine cell
40 ELISA, enzyme linked immunosorbent assay

41 FACS, fluorescence activated cell sorting
42 FFA1,2, free fatty acid receptors 1,2
43 GI, gastrointestinal
44 GIP, glucose-dependent insulinotropic peptide
45 GLP-1, glucagon-like-peptide-1
46 GLP1R, GLP-1 receptor
47 Glu, glucagon (promoter)
48 GLUT, glucose transporter
49 GPBAR1, G-protein coupled bile acid receptor
50 GPCR, G-protein-coupled receptor
51 IBMX, 3-Isobutyl-1-methylxanthine
52 INSL5, insulin-like-5
53 NTS, neurotensin
54 PYY, peptide YY
55 RIA, radioimmunoassay
56 SCFA, short chain fatty acids
57 SGLT, sodium glucose linked transporter
58 VGCC, voltage, gated calcium channels
59
60
61
62
63
64 The gastrointestinal (GI) tract epithelium is produced by stem cells that differentiate into
65 absorptive and secretory cell types including the rare hormone producing enteroendocrine
66 cells (EECs). Taken as a whole, EECs make up the largest endocrine system in the body, with
67 over 20 different gut hormones having been described. Classically EECs have been defined
68 by which hormones they express and secrete (Figure 1), although it is now evident that
69 individual EECs exhibit an unexpected degree of heterogeneity in hormone expression (1).
70 Gut hormones play a number of roles in normal and disease physiology and are a major
71 focus of academic and industrial research. The GI tract is exposed to ingested nutrients,
72 circulating hormones/nutrients, and other GI components such as bile acids and bacterial
73 metabolites. Many of these act as physiological stimuli that promote or inhibit hormone
74 secretion from EECs (Figure 1). Once secreted, gut hormones can act locally in a paracrine
75 manner and target other cells in the mucosa and local neuronal networks, or enter the
76 bloodstream to reach distant organs. In this review we will highlight the different
77 experimental models and tools used for EEC research.
78
79 **Model Tissues for Enteroendocrine Research**

80 There are many models currently being used to investigate EECs. In this review we will
81 concentrate on ex vivo and in vitro models. The majority of research in the field has been
82 performed using animal tissues (mostly rodent and in particular murine) as these are readily
83 available, relatively inexpensive, and make use of the multiple transgenic strains available.
84 Ideally for research into the role of EECs in health, human tissue would be used, but this is
85 restricted by tissue availability, practicalities and additional technical difficulties. Some
86 studies have used pigs, as at is has been suggested they are a more relevant model for
87 humans; unlike rodents, for example, they express the hormone motilin. However, there are
88 key digestive differences between the two species, including increased GI fermentation in
89 pigs which is likely to influence EEC hormone secretion. Pigs also have the disadvantage over
90 rodents that due to their size, studies require larger facilities, more labour time and
91 increased animal housing costs.
92
93 Here we will discuss ex vivo model systems involving isolated intestinal perfusion and Ussing
94 chambers, and in vitro models such as cell lines, short term primary cultures and intestinal
95 organoids (Figure 2). The characteristics of each model with regard to throughput capability,
96 tissue suitability, longevity etc have been summarised in Figure 2a.
97
98
99 ***Ex vivo* Studies**
100 Isolated intestinal perfusion and Ussing chamber systems are classic ex vivo models that
101 have been widely used to investigate EEC function in different regions of the GI tract (2-10).
102 Intestinal perfusion can be performed on animals as small as mice, and generally involve
103 isolating a target gut segment whilst it is physically retained within the abdominal cavity,
104 enabling vessel cannulation whilst the heart is still beating. The gut segment under
105 investigation is defined by the area supplied by the cannulated artery, and the lumen can be
106 additionally cannulated and perfused (Figure 2c). In further modifications, the method has
107 been adapted to enable gut hormone sampling from the intestinal lymph (11). Perfused
108 intestinal models can be used to monitor hormone secretion from different regions of the
109 small intestine or the colon, and are a useful tool to study EECs in the context of their
110 surrounding environment, as their local neuronal and vascular circuits largely remain intact,
111 thus supporting near normal regulation of the EECs. The intestinal epithelial barrier is also

112 maintained, allowing luminal and systemic application of nutrients or drugs to identify their
113 directionality of action. A blood substitute without red blood cells is commonly used for the
114 vascular perfusion, allowing larger sample volumes and more frequent sampling than is
115 possible in in vivo studies on mice, and thus better resolution of hormone secretory
116 dynamics. Hormone secretion has also been measured from isolated intestinal loops
117 mounted in liquid paraffin, which can be readily perfused via the gut lumen but not through
118 the vasculature. This method relies on the detection of hormones after they have diffused
119 through the muscular layers and serosa into the surrounding space where they collect in
120 small aqueous pools adjacent to the paraffin (12). Although this is a simpler experimental
121 setup than the vascularly perfused intestine, hormone diffusion is too slow to allow
122 resolution of the time course of secretion, and the tissue is more prone to hypoxia due to
123 the lack of continuous vascular perfusion with oxygenated medium. For obvious ethical
124 reasons, isolated human intestinal perfusion studies are not possible, and most research is
125 performed in rodents, or sometimes in pigs as they are considered a more human relevant
126 model. Isolated intestinal perfusion studies involve highly specialised surgical techniques
127 and are of low throughput compared with the in vitro models which are quicker, easier and
128 can be scaled up to enable the parallel examination of multiple test conditions.
129
130 Ussing chambers have also been extensively used to study hormone secretion from intact
131 intestinal tissue pieces (2,6-10). This method involves mounting tissue segments in
132 specialised chambers that were classically used to investigate mucosal physiology and
133 pharmacology (Figure 2d). As with the perfused intestinal models, the epithelial barrier is
134 maintained, enabling questions to be asked about whether drugs and nutrients target EECs
135 from the luminal or basolateral direction (2,10). In this system the integrity of the tissue
136 (epithelial barrier) can be assessed using electrodes which enable live assessment of the
137 trans-epithelial resistance and potential difference of the mounted specimen. Hormone
138 secretion in Ussing chamber studies can be monitored either by directly assaying hormone
139 concentrations in the basolateral chamber, or by measuring the electrical properties of the
140 epithelium which are altered when secreted hormones such as PYY and GLP-1 target their
141 receptors on enterocytes or enteric neurones. Electrophysiological changes in response to
142 cAMP elevation through forskolin/IBMX can also be used to assess the
143 responsiveness/viability of the investigated tissue at the end of the experiment. When

144 hormone concentrations are measured in Ussing chambers by immuno-assay, optimisation
145 of the chamber volumes and tissue size is required to ensure that hormone concentrations
146 are not below the detection limit of the assay. It is also sometimes necessary to strip away
147 the muscle layers to improve diffusion of hormones and stimuli between the EECs and the
148 basolateral fluid compartment.
149
150 A significant drawback of both these ex vivo systems is the limited experimental window
151 during which the tissue remains healthy. Unlike in vitro models which can be kept for a few
152 days in primary culture or maintained indefinitely as cell lines, the ex vivo model systems
153 described above remain viable for only a few hours. Upper small intestinal tissue mounted
154 in an Ussing chamber, for example, has been shown to become compromised after just 2
155 hours, although more distal regions were slightly more resilient (13). This has contributed to
156 a paucity of Ussing chamber studies examining EECs in the upper GI tract. Ussing chambers
157 have been used to study human as well as mouse EECs, as the method only requires small
158 tissues pieces that can be obtained under ethical approval from routine surgical operations
159 that resect normal tissue as part of a clinical procedure. Nevertheless, studies on human
160 intestine are limited by tissue availability and by the integrity of the epithelium, as human
161 samples generally incur significant delays between tissue collection and experimentation
162 (14).
163
164
165 **In Vitro Studies**
166 Historically, a basic cellular tool for many researchers has been cell lines - immortalised cells
167 that can be routinely passaged and maintained, and scaled up for high throughput studies
168 or experiments requiring large cell numbers. There are multiple cell lines used as models for
169 EEC research, including GLUTag, NCI-H716, STC-1, HuTu-80 and BON cells. Several cell lines
170 have been used as models for GLP-1 producing L-cells, including GLUTag, a murine
171 endocrine tumour adherent cell line, STC-1, a rat adherent cell line and NCI-H716, a human-
172 derived suspension cell line (15-18). Multiple disadvantages are associated with the use of
173 cell lines in research: many lines are derived from tumours and therefore have genetic and
174 morphological differences from their in vivo counterparts, they are a simplified system and
175 grown without other cell types present, and they lack many physiological traits of their

176 native counterparts with regards to cell morphology and polarity. At the transcriptomic level,
177 GLUTag and STC-1 cells exhibited many similarities to native L-cells, but also a number of
178 differences, including altered expression of key G-protein coupled receptors involved in L-
179 cell sensing (19,20). A recent analysis of different EEC cell lines also found substantial
180 differences in hormone processing compared with native cells (18). Cell lines do, however,
181 play an important role in EEC research, particularly for studies that require high cell
182 numbers or high throughput.
183 In the last decade, techniques and protocols have been developed for the use of primary
184 EECs in research. A number of groups have measured hormone secretion from intestinal
185 tissue biopsies, but responses to stimuli are relatively weak in this setting (14,21). In 2008,
186 Reimann et al published the first protocol for the use of short lived (days) 2-dimensional (2D)
187 primary cultures of the murine intestinal epithelium for studying EECs, which has recently
188 been published as a video protocol ((22,23), Figure 2f). This method enabled, for the first
189 time, assessment of primary L-cells by electrophysiology and calcium imaging techniques,
190 combined with measures of hormone secretion. Since publication, this technique has been
191 successfully applied to intestinal tissues from other species including human, and has
192 supported the identification of many nutrient- and drug-activated pathways involved in the
193 release of gut hormones from varying regions along the GI tract (2,6,7,20,24-26). Typical
194 preparations from mouse or human tissue samples generate approximately one 24-well
195 plate per 5-10cm length of mouse intestine, so this method allows several drug treatments
196 to be tested in parallel, giving a relatively high experimental throughput compared with ex
197 vivo studies. Whilst the protocol can potentially be scaled up to 96-well plates or beyond,
198 the method is ultimately limited by the sensitivity of the immuno-assays used for hormone
199 detection. The major drawbacks of this system are the short-lived nature of the culture, the
200 isolation of EECs from some of the surrounding cell types, loss of apico-basal polarity and
201 lack of the epithelial barrier. In cultures derived from the small intestine, EECs only survive
202 for a few days, whereas EECs from the colon were still functional after 10 days (23). No new
203 EECs appear to form under these culture conditions (22,23) so every preparation requires a
204 fresh tissue sample, incurring associated animal costs and local availability of suitable
205 mouse strains. Because these intestinal primary cultures do not generate an epithelial
206 barrier or exhibit apico-basal polarity, they are not suitable for analysis of whether stimuli
207 act via the apical or basolateral surface.

208

209 Once EECs have been completely separated from their neighbours by enzymatic or EDTA
210 dispersal, they seem not to survive for long in culture, although calcium imaging was
211 successfully performed in acutely isolated CCK-producing cells by flow cytometry based on
212 their cell specific expression of GFP (27). By contrast, Raghupathi et al (2013) separated
213 enterochromaffin cells, an EEC type that primarily secretes serotonin (Figure 2e), using a
214 Percoll density gradient and generated a 95% pure population of enterochromaffin cells that
215 could be kept in culture for up to 4 days with a 70% survival rate (28-30). The
216 enterochromaffin cells isolated by this process have been assessed using amperometry, rt-
217 qPCR and calcium imaging. The drawbacks of this technique are similar to those described
218 for primary intestinal cultures with the added uncertainty of the potential functional
219 consequences of separating EECs from their neighbouring enterocytes.

220

221 The use of GI organoids rather than primary cultures eliminates the need for a constant
222 supply of freshly-harvested tissue (Figure 2g). Intestinal organoids are 3D structures grown
223 from stem cells, and consist of organ-specific cell types that self-organise and show spatially
224 restricted lineage commitment (31). These organoids are initiated from either pluripotent or
225 adult stem cells and are grown in specialized media that mimic the stem cell niche.
226 Protocols to generate intestinal organoids from embryonic or induced pluripotent stem cells
227 are under development, but still require refinement to optimise the differentiation and
228 maturation of EECs (32,33). It is relatively straight-forward, however, to produce organoids
229 from adult intestinal stem cells, which are coerced to form organoids by creating conditions
230 that mimic the stem cell niche environment deployed during tissue self-renewal or damage
231 repair. Indeed, the intestine is a self-renewing tissue containing abundant adult crypt stem
232 cells, and was one of the first tissues to be used to generate long term organoids from
233 mouse and human (34,35). 3D organoids can be maintained in culture by regular splitting
234 and re-plating, and can be cryopreserved in much the same way as a standard cell line.
235 Depending on the intestinal region and species of origin, however, it is often necessary to
236 change the culture conditions to promote the formation of fully differentiated EECs, and
237 once they have been induced to form a terminally differentiated state containing functional
238 EECs, organoid cultures generally have a limited survival of up to a week, thus resembling
239 the behaviour of primary cultures derived from freshly-harvested tissue.

240

241 Intestinal organoids have recently been used to investigate EEC function and represent a
242 promising model for EEC research (36,37). When generated from transgenic mouse models
243 expressing fluorescent sensors and reporters driven by different gut hormone promoters,
244 for example, they provide a replenishable source of identifiable EECs for single cell analysis.
245 Organoid cultures have been shown to contain different types of EECs and to retain their
246 regional identity with regard to the profile of gut hormones produced (38). Other studies
247 have shown that EECs generated within intestinal organoids are responsive to a range of
248 physiological stimuli and are secretion competent (36,37,39,40). Cells within 3D organoids
249 have apico-basal polarity with their apical membranes located towards the organoid core.
250 Apical surfaces are therefore largely inaccessible when stimuli are applied via the media,
251 which is a potential limitation of the model as most nutritional stimuli first reach their target
252 EECs in vivo via the gut lumen. However, experiments using dextran labelling have
253 suggested that organoids are leaky and that solutions can readily reach the apical
254 membrane (40). We recently developed a 2D plating method for intestinal organoids to
255 facilitate patch clamp electrode access, cellular imaging within a single focal plane and
256 measurements of GLP-1 secretion (36). Data from these studies showed that L-cells derived
257 from murine organoids exhibited similar responses to L-cells in ileal primary cultures, thus
258 validating the system as a good model for EEC research. This method is particularly exciting
259 because organoids, like cell lines, can be genetically modified to produce lines with
260 knockout, knockin and overexpression of genes of interest. Similar genetic manipulations of
261 human derived organoids would give this technique unique advantages compared with
262 other ex vivo and in vitro models.

263

264 Interestingly, recent progress has been made in converting 3D organoid cultures to an open
265 faced polarised epithelium that can be used for transport studies (41,42). One such
266 technique has been shown to recapitulate the normal intestinal hierarchy and morphology,
267 with the formation crypt/villus structures resembling in vivo tissue architecture (41). This
268 method combined with genetically modified organoids will potentially open up new
269 avenues for EEC research that are not currently possible with existing techniques.

270

271

272 **Experimental Tools for EEC Research**

273

274 **Hormone Secretion Studies**

275 The experimental models described above have been developed to study the mechanisms
276 regulating hormone secretion in response to application of drugs or physiological stimuli.
277 There is widespread interest in this field, because gut hormones have a variety of
278 physiological roles, are currently exploited therapeutically for the treatment of type 2
279 diabetes and obesity, and are under evaluation as future drug targets for a variety of
280 metabolic and GI conditions. Hormones secreted by EECs have been measured by enzyme
281 linked immunosorbent assays (ELISA), radioimmunoassays (RIA), mass-spectrometry,
282 amperometry and by their effects on epithelial short circuit currents.

283

284 Immuno-assays use antibodies directed against target peptides, linked to quantifiable
285 outputs such as enzymatic reactions, radioisotopes or electroluminescent signals that are
286 related to the quantity of antibody bound to the target peptide. The best immuno-assays
287 have a very high sensitivity, which is essential for the analysis of gut hormone
288 concentrations that typically range from 1-100 pg/ml in plasma. Nevertheless, an immuno-
289 assay is only as good as its component antibodies, which must have both high affinity and
290 high specificity. These are typically tested by cross-reactivity experiments with other
291 peptides as well as standard curve analysis using serial dilutions of target peptides. ELISAs
292 have been successfully used to measure GLP-1, GIP, SST, neurotensin, serotonin and PYY
293 secretion from a variety of ex vivo and in vitro models (4,6,23,26,28,43). Drugs which
294 directly target known secretory signalling pathways, including cAMP (IBMX, forskolin) and
295 protein kinase C (PMA) are typically used as positive controls in hormone secretion
296 experiments.

297

298 As discussed above, individual EECs can produce multiple hormones, and an ideal detection
299 method would therefore enable the parallel measurement of multiple hormones from the
300 same sample. This can be achieved by multiple or multiplexed ELISAs, but the performance
301 of multiple assays on the same sample is generally limited by cost and sample volumes, and
302 many multiplexed assays still perform less well than when individual hormones are assayed
303 separately. The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) for

304 the detection of peptide hormones is an emerging field that enables the parallel detection
305 of multiple hormones from a single sample (44,45). This is particularly useful for hormones
306 such as INSL5, for which there are currently no reliable commercial immunoassays available
307 (46). Using LC-MS, we could detect INSL5 secretion from primary human and mouse colonic
308 cultures (44), and have simultaneously measured GLP-1, PYY and INSL5 release from human
309 and mouse colonic cultures in response to a variety of drugs and physiological stimuli (47).
310 LC-MS/MS analysis of hormone levels does come with some caveats compared to traditional
311 ELISAs, including generally lower sensitivity and the need for modified peptides to be spiked
312 into each sample to both account for loss during preparation and to accurately quantify the
313 relevant hormone levels.
314
315 Serotonin secretion from isolated enterochromaffin cells has been measured by ELISA or
316 carbon fibre amperometry (14,28,29). Amperometry enables the detection of individual
317 vesicular release events which trigger discrete currents in the carbon fibre electrode due to
318 the oxidation of serotonin (29,48).
319
320
321 **Transgenic Mice**
322 The generation of transgenic reporter mice has enabled a dramatic increase in our
323 understanding of EEC function and has helped to identify and validate a wide range of
324 signalling pathways controlling hormone secretion from the intestine. Most genetically
325 modified mouse models used in EEC research harness cell-specific promoters, such as the
326 promoters for the hormones themselves (e.g. GLP-1, GIP, CCK, SST, ghrelin), chromogranin A,
327 EEC-specific transcription factors (e.g.NeuroD1) or tryptophan hydroxylase (Tph1, required
328 for serotonin biosynthesis) (Figure 3) (23,26,43,49-57). These EEC-specific promoters are
329 used to drive cell-specific expression of fluorescent reporters/sensors or Cre-recombinase.
330 Interestingly, the Chga-hrGFP reporter line seems specifically to label enterochromaffin cells
331 even though ChgA is considered to be a marker of most EECs, although this mirrors our
332 previous observation that ChgA staining was low in murine L-cells compared with other EECs
333 (37,56).
334

335 When the fluorescent reporter is directly driven by a hormonal promoter, the detection of
336 fluorescence in a cell should reflect current or recent activity of the hormonal promoter, as
337 is seen with mouse strains such as GLU-Venus, GIP-GFP, CCK-GFP and ChgA-hrGFP
338 (23,52,56,58). Hormone promoters driving Cre-recombinase, by contrast, are actually
339 lineage tracers in the sense that even a brief activation of the hormone promoter and
340 consequent transient expression of Cre-recombinase can activate Cre inducible elements in
341 the DNA, causing permanent genetic changes that persist for the lifetime of the cell and are
342 passed on to daughter cells. If Cre-expression is used to label an EEC population, it does not
343 therefore necessarily reflect ongoing hormone expression. This can be useful when the
344 endogenous cell-specific promoter is too weak itself to drive detectable fluorescence,
345 because it is used instead to activate a fluorescent reporter that is driven by a much
346 stronger promoter. Although, as with all indicators, one might be concerned that chronic
347 overexpression of fluorescent reporters might be toxic or alter the dynamics of the
348 monitored secondary messengers, we have not detected any differences in the
349 responsiveness of our reporter lines to different secretory stimuli and observed similar Ca^{2+}-
350 dynamics when monitoring with GCaMP3 or Fura-2.
351
352 Transgenic mouse technologies have allowed researchers to identify the rare EEC cells
353 amongst the rest of the intestinal epithelial cells, enabling application of a wide variety of
354 analytical methods, including population and single cell transcriptomics, electrophysiology
355 and single cell imaging of intracellular signalling pathways.
356

357 **Flow cytometry**

358 EECs expressing fluorescent reporters, derived from transgenic mouse models, can be
359 readily separated from their non-fluorescent neighbours by fluorescence-assisted cell
360 sorting (FACS) following dispersal of the mucosa by enzymatic digestion or EDTA (23). Flow
361 cytometry gates set on forward scatter and side scatter enable the distinction between cells
362 and debris, and an additional pulse width gate is useful to reduce the collection of cell
363 clusters containing a single fluorescent EEC that appear on the FACS as a single large
364 fluorescent cell. EEC populations of 90-95% purity can be achieved by FACS, with the major
365 limiting feature being the quality of the single cell digest. Additional nuclear stains are
366 frequently used to exclude dead cells.

367

368 Flow cytometry can also be used to analyse the proteins produced by individual EECs, in a
369 method that involves fixation and antibody staining of cell suspensions following tissue
370 dispersal (19). The technique enables the identification and quantification of EECs producing
371 e.g. different hormonal combinations, or other high abundance proteins that are readily
372 detectable using antibodies. We have also developed protocols to enable transcriptomic
373 analysis of fixed and stained EECs following FACS purification, allowing the characterisation
374 of non-murine species, including human, that do not carry transgenic markers (Roberts et al,
375 manuscript under review). A similar approach has recently been reported for the isolation of
376 murine chromaffin cells not relying on transgenic labelling (59).

377

378 **Transcriptomics**

379 FACS purified EECs have been analysed at the transcriptomic level by rt-qPCR to quantify
380 pre-selected transcripts, and by microarrays and RNA seq to identify all transcripts. RNA
381 sequencing methods are now sufficiently sensitive to permit analysis of single cells as well
382 as cell populations, although single cell analysis is still limited by read-depth and the costs of
383 analysing large numbers of cells individually. By these methods, it has been shown that
384 primary EECs express a wide variety of G-protein coupled receptors (GPCRs), transporters
385 and channels which have subsequently been shown to play roles in the function and
386 physiology of EECs (23,37,60). Application of single cell RNA sequencing methods to
387 investigate EECs was first achieved by random sampling of intestinal organoid cells (61) or
388 primary cells (1) and more recently was applied to duodenual L-cells purified by FACS from
389 the GLU-Venus mouse model (58). These data have highlighted the heterogeneity of EECs
390 with regard to expression of multiple hormones and GPCRs. Enterochromaffin cells in
391 organoids labelled with the ChgA-hrGFP reporter have also been investigated by rt-qPCR
392 and RNA sequencing, to identify the channels/transporters and receptors that are
393 differentially expressed compared with the non-fluorescent cell population (37).

394

395 **Electrophysiology**

396 Electrophysiology evaluates the flow of ions or potential difference across biological tissues,
397 or cell membranes. In Ussing chambers, the short circuit current can be used as a measure
398 of net ion transport across the epithelium and has been used to assess PYY secretion

399 triggered by nutrients, hormones and drugs (6,9). Locally released PYY binds to NPY1R
400 receptors on enterocytes, thereby lowering intracellular cAMP concentrations and blocking
401 apical chloride secretion which can be measured by changes in the short circuit current (8).
402
403 Early patch clamp studies of STC-1 cells revealed the presence of voltage gated Ca^{2+} and K^+
404 currents, but did not find any direct evidence that the cells fired action potentials (62).
405 GLUTag cells, and EECs in primary cultures and organoids, by contrast, were found to be
406 electrically active and capable of firing action potentials dependent on voltage gated Na^+
407 and Ca^{2+} channels (23,36,37,60,63-67). The detection of electrical activity in GLUTag and
408 primary L-cells likely reflects the use of perforated patch rather than whole cell recordings
409 which retain a more physiological intracellular milieu. Indeed, EECs exhibit very high
410 electrical resistance, and only small leak currents are sufficient to abolish action potential
411 firing. Whilst patch clamping of colonic primary L-cells (identified using GLU-Venus mice)
412 and GLUTag cells is relatively straight forward because colonic L-cells survive in culture for
413 over a week (64,67), we found it considerably more difficult to patch clamp duodenal EECs
414 in primary culture because they only survived for a few days, and produced substantial
415 quantities of mucus that tended to block the electrodes. Organoid-derived ileal L-cells,
416 however, proved a more robust system for patch clamping, revealing that ileal L-cells like
417 their colonic counterparts fire action potentials that are modulated by external stimuli (36).
418 Organoid-derived enterochromaffin cells from ChgA reporter mice also exhibited Na^+ and
419 Ca^{2+} dependent action potentials (37).
420
421 **Live cell imaging**
422 Fluorescent dyes and genetically encoded sensors have been used to monitor intracellular
423 concentrations of Ca^{2+}, cAMP and glucose in EECs in response to stimulus application. Cell
424 permeant Ca^{2+} indicators such as Fura2-AM can be used to load an entire dish of cells, and
425 have been employed to monitor Ca^{2+} in a variety of EEC cell lines (67-69). For primary EECs,
426 however, extra steps must be incorporated that permit EEC identification amongst the
427 mixture of other cell types, or include prior cell purification. CCK-expressing cells, for
428 example, have been FACS purified from CCK-eGFP mice and loaded with the Ca^{2+} indicator
429 Quest Rhod4, to show that CCK-producing cells exhibited Ca^{2+} responses to ligands of the
430 free fatty acid receptor FFA1 (70), and enterochromaffin cells were purified by Percoll

431 gradients prior to Fluo-4 loading, to enable Ca^{2+} imaging of the enterochromaffin cell
432 population (28-30). More recently, however, we have seen the introduction of genetically
433 encoded sensors that incorporate a binding domain for the signal of interest, fused to one
434 or more fluorescent molecules that exhibit altered fluorescence intensity or fluorescence
435 resonance energy transfer (FRET) upon ligand binding (25,50,71). Genetically encoded
436 sensors provide the advantage that they can be specifically expressed in the target cell of
437 interest using transgenic technologies. ROSA26-GCaMP3 reporter mice, for example,
438 express the Cre-dependent Ca^{2+} sensor GCaMP3, and can be crossed with mouse strains
439 expressing Cre-recombinase under EEC-specific promoters to enable Ca^{2+} recordings in EEC
440 populations of interest. Most genetic sensors have not, however, yet been incorporated into
441 Cre-dependent mouse strains, but can be introduced into cell lines by standard transfection
442 methods to enable monitoring of intracellular cAMP or glucose concentration (50,71). Use
443 of these sensors in primary EECs requires an additional method for cell identification, such
444 as a transgenic EEC-specific fluorescent reporter activated at wavelengths that do not
445 interfere with the sensor, or prior cell purification by FACS. Expression of the genetic sensor
446 can also be driven directly by an EEC promoter in a transgenic mouse model, as exemplified
447 by GLU-Epac2-camps, which expresses the cAMP sensor Epac2-camps driven by the
448 proglucagon promoter to allow cAMP monitoring in L-cells (49). These methods have
449 enabled dynamic monitoring of GPCR signalling pathways in live EECs following stimulus
450 application, assessment of net glucose fluxes, and Ca^{2+} changes downstream of Ca^{2+} influx
451 or release from intracellular stores.
452
453
454 **Assessing physiological EEC stimuli by combined experimental techniques**
455 The models and experimental tools described above have been used to identify a wide
456 range of stimulants and suppressors of gut hormone release, site of interaction of nutrients
457 with EECs and some of the molecular pathways involved in nutrient detection and hormone
458 secretion. Many of the findings from these studies are summarised in Figure 4. As an
459 example of how the different techniques have been combined to explore a molecular
460 pathway, we will consider the question of how bile acids trigger GLP-1 secretion.
461

462 Bile acids have been demonstrated to increase GLP-1 secretion from GLUTag cells, primary
463 colonic L-cells, ileal organoid-derived L-cells, ileal tissue mounted in Ussing chambers and
464 perfused intestine (2,5,10,36,72). Knock-down studies in GLUTag cells and the use of tissues
465 from knock-out mice confirmed that the major receptor involved in the bile acid triggered
466 GLP-1 secretion is the G-protein coupled bile acid receptor GPBAR1 (also known as TGR5),
467 which is highly expressed in primary L-cells (2,72,73). Consistent with the known $G\alpha s$
468 coupling of GPBAR1, bile acids and the GPBAR1 agonist GPBAR-A increased intracellular
469 cAMP levels in GLUTag cells transiently expressing the cAMP FRET sensor Epac2-camps, as
470 well as in primary L-cells in organoids derived from the GLU-Epac2-camps mouse model
471 (2,36,72). The use of 2D organoid cultures allowed electrophysiology assessment of the bile
472 acid receptor in ileal L-cells, revealing that GPBAR1 agonism increased action potential firing
473 and enhanced the activity of voltage gated Ca^{2+} channels, thereby potentiating Ca^{2+} signals
474 triggered by FFA1 agonists, as measured in organoid L-cells from GLU-Cre/GCaMP3 mice
475 (36). A key question for the potential translational exploitation of GPBAR1 as a drug target
476 for increasing endogenous GLP-1 secretion is whether small molecule receptor agonists
477 could target the receptor from the apical membrane, thus enabling the development of
478 non-absorbable agonists with low side effect profiles. Ussing chamber and perfused
479 intestine experiments, however, demonstrated that bile acids only enhance GLP-1 secretion
480 following their absorption across the epithelium, and that non-absorbable GPBAR1 agonists
481 are only effective when applied from the basolateral direction (2,5,10). Nevertheless,
482 activation of GPBAR1, particularly in combination with FFA1, provides strong stimulus to L-
483 cells that deserves further exploration as a potential therapeutic strategy.
484

485 **Concluding Comments**
486

487 Compared with the situation 20 years ago, when studies on gut hormone secretion were
488 largely restricted to the use of whole animals, perfused intestinal preparations, Ussing
489 chamber recordings and cell lines, EEC research has exploded recently both in terms of the
490 techniques and protocols available to researchers and our understanding of the molecular
491 pathways employed by EECs for stimulus secretion and detection. The use of high
492 throughput systems such as cell lines and primary cultures has enabled the identification of
493 a wide range of nutrient and non-nutrient dependent pathways involved in the stimulation

494 and inhibition of hormone release. Increased use of intestinal organoids is hoped to
495 facilitate more translational human based research and reduce the need for ex vivo animal
496 based studies. The new technologies that use organoid cultures to generate an in vivo type
497 intestinal architecture with intact barrier function provide promising new avenues for the
498 study of human and murine EEC function.

499

500 **Figure legends**

501 *Figure 1: Enteroendocrine cells and their hormones*

502 (a) Table listing the different EEC types and the main hormones/monoamines they produce.
503 (b) Schematic showing an open-type EEC (blue) facing into the gut lumen, between
504 neighbouring enterocytes in the intestinal epithelium, highlighting sites of stimulus
505 detection and hormone (red) secretion.

506

507 *Figure 2 Ex vivo and in-vitro models for studying enteroendocrine cells*

508 (a) Table showing the different features and applications of ex vivo (isolated perfused
509 intestine and Ussing chambers) and in vitro (primary cultures, isolated EECs, organoids)
510 models described in the text. (b) Schematic of small intestine and colonic epithelium with
511 the rare EECs (blue) found in both crypts and mature epithelium (villi and surface
512 epithelium). (c-g) Diagrams showing the different model systems. (c) In isolated perfused
513 intestine, solutions can be perfused via the intestinal lumen and vascular supply. (d) Ussing
514 chamber setup, in which tissue forms a barrier between 2 compartments (integrity measure
515 by electrodes). Solutions can be added to, and sampled from, either compartment. (e)
516 Single cell digest of intestinal epithelium, enabling EECs to be isolated by FACS or Percoll
517 density gradient. (f) Primary intestinal monolayers generated by epithelial digestion and
518 seeding onto a matrix scaffold (black) to form a non-polarised monolayer in which EECs are
519 intermixed with other epithelial cell types. (g) 3D intestinal organoid structures resembling
520 in vivo architecture (crypt and villi domains) with polarised epithelium containing all cell
521 types including EECs.

522

523 *Figure 3: Transgenic enteroendocrine cell models*

524 (a) Table listing the current transgenic models used to identify different EEC types. Glu-
525 Venus (green) mouse tissue showing colonic epithelium (b, image by L.Billing), an isolated

526 ileal villus (c) and isolated ileal crypts (d). Ileal organoid derived from Glu-Epac2-camps mice
527 (e), with L-cells highlighted by Epac2-camps expression (green). Scale bar 100μm
528
529 *Figure 4: Mechanisms underlying glucose, SCFA, bile acid and amino acid sensing in*
530 *enteroendocrine cells.*
531 (a) Glucose sensing in EECs, mediated by 2 pathways leading to cell depolarisation and
532 opening of voltage gated calcium channels (VGCC), triggering Ca^{2+} influx and hormone
533 secretion. Glucose co-transport with Na^{+} through apical SGLT1 can trigger depolarisation
534 and secretion, whereas glucose influx via GLUT2 increases ATP levels and closes K_{ATP}
535 channels, potentially modifying the effectiveness of other depolarising stimuli. (b) SCFA
536 sensing in EECs mediated by FFA2 Gαq signalling, thereby increasing Ca^{2+} and triggering
537 hormone release. An unidentified pathway might also trigger EEC depolarisation and
538 opening of VGCC, as suggested by experiments on perfused intestine. (c) Bile acid sensing is
539 mediated by basolateral GPBAR1 signalling through Gαs, increasing cAMP and triggering
540 hormone secretion. GPBAR1 activation also promotes depolarisation and opening of VGCC.
541 (d) Amino acid sensing in EECs is mediated by multiple pathways. Highlighted here are CASR
542 Gαq signalling and electrogenic amino-acid uptake which trigger subsequent depolarisation
543 and opening of VGCCs.
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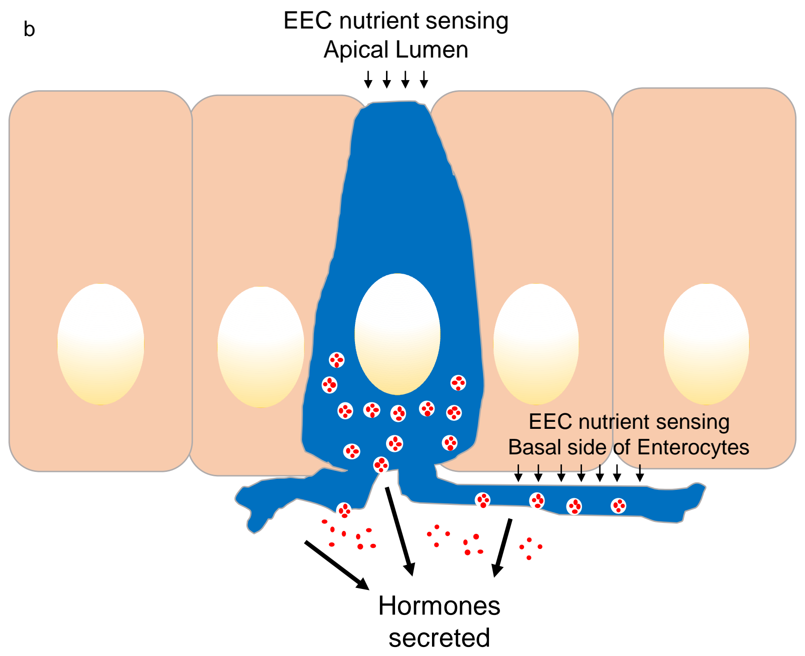
Cell Types	Main Hormones / Monoamine Secreted
L-cells	GLP-1, GLP-2, PYY
K-cells	GIP
I-cells	Cck
D-cells	Somatostatin
EC	Serotonin
N-cells	Neurotensin
M-cells	Motilin
S-cells	Secretin
X/A like	Ghrelin
ECL-cells	Histamine
G-cells	Gastrin



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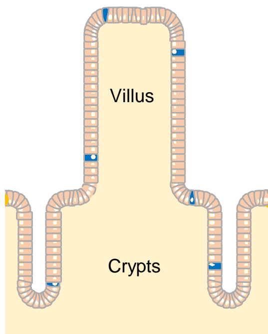
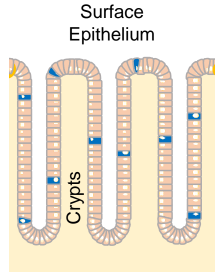
	High Throughput	Hormone Secretion Analysis	Single Cell Analysis	Epithelial Barrier	Polarity	Cell-cell Contacts	Neuronal / Blood Connection	Rodent Tissue	Human Tissue	Long Term (self renewing)	Experiment Time Window
Isolated Perfused Intestine		x		x	x	x	x	x			hrs
Ussing Chambers		x		x	x	x		x	x		hrs
Primary Monolayer	x	x	x			x		x	x		days
Isolated EECs	x	x	x					x			days
Organoids	x	x	x		x	x		x	x	x	years
Organoid derived monolayer	x	x	x			x		x	x		days

b

Small Intestine

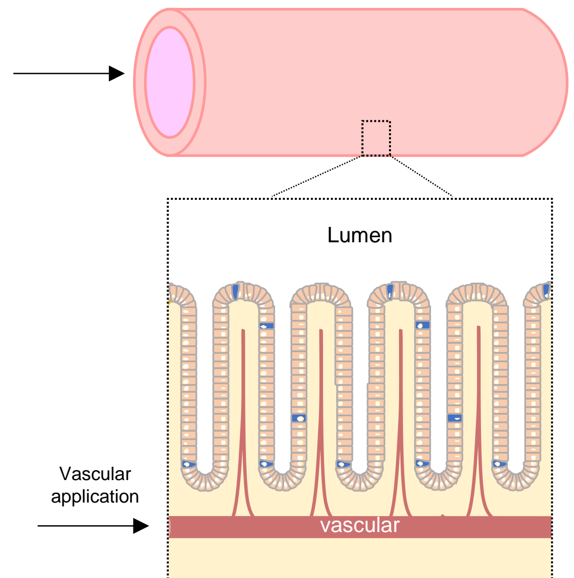


Colon



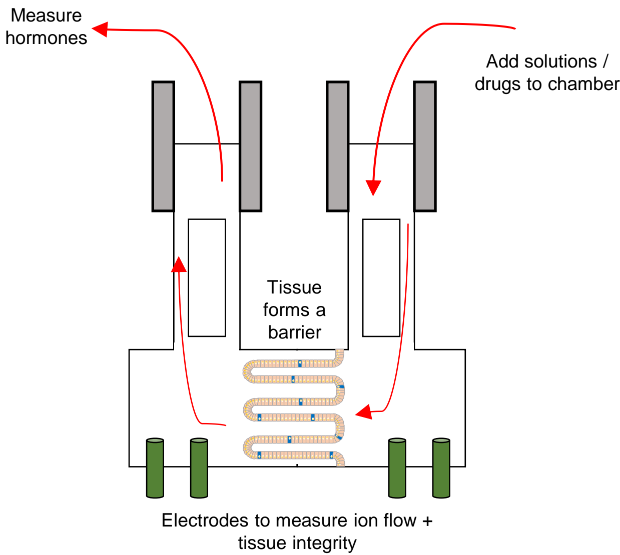
c

Isolated Intestinal Perfusion



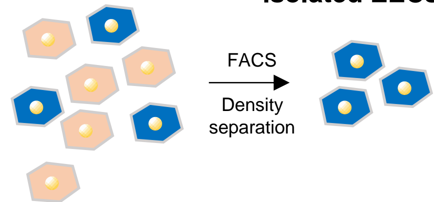
d

Ussing Chambers



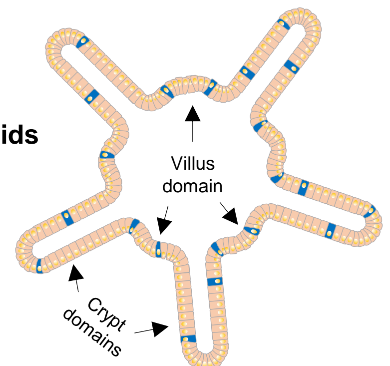
e

Isolated EECs



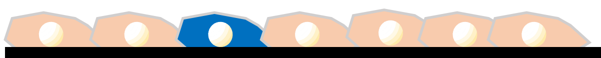
g

Organoids



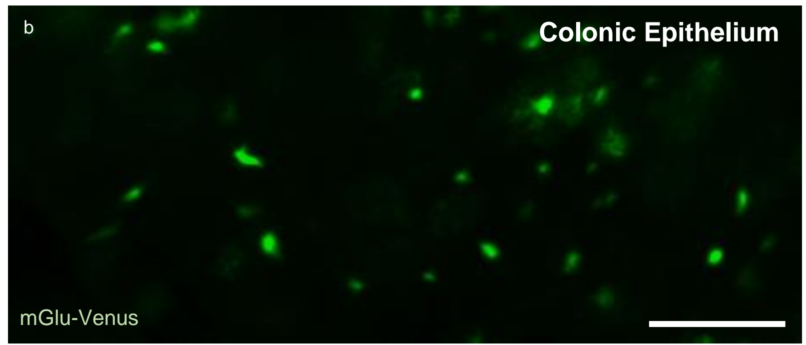
f

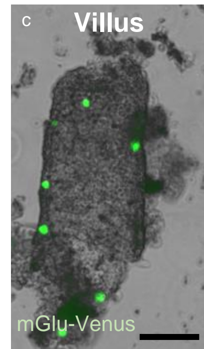
Intestinal Monolayers

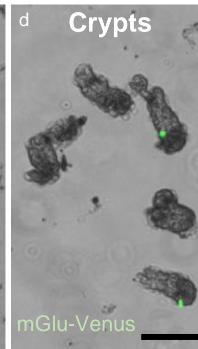


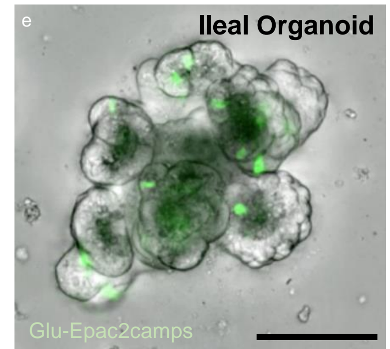
a

Hormone Promoter Targeted	Cell-Type	Transgenic model
ChgA	EC-cells	ChgA-hGFP (56)
GLP-1	L-cells	mGlu-Venus (23)
		Glu-Epac2-camps (49)
		Glu-Cre (50)
GIP	K-cells	Gip-Venus (26)
		Gip-GFP (52)
		Gip-Cre (51)
CCK	I-cells	Cck-GFP (53)
SST	D-cells	Sst-Cre (43)
5-HT	EC-cells	Tph1-CFP (54)
Ghrelin	X/A like	Ghrelin-hrGFP (55)
PYY	L-cells	PYY-GFP (57)

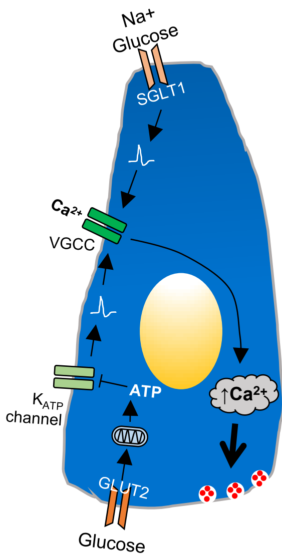








a



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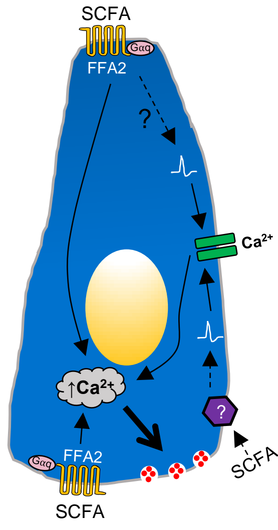
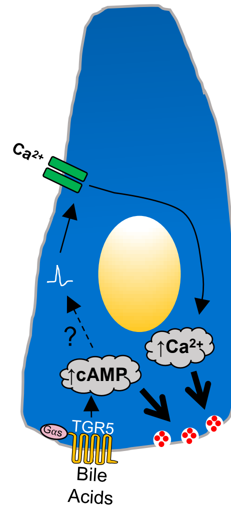


c



d