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Serotonin release and uptake in the gastrointestinal tract

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Abbreviations used in this paper: gastrointestinal (GI); enteric nervous system (ENS); cholecystokinin (CCK), adenosine-5'-triphosphate (ATP), enterochromaffin (EC) cell; enteroendocrine (EE) cell; ulcerative colitis (UC); irritable bowel syndrome (IBS); inflammatory bowel disease (IBD); serotonin (5-hydroxytryptamine or 5-HT); serotonin reuptake transporter (SERT); tryptophan hydroxylase (TpH); 5-hydroxytryptophan 5-HTP; 5-hydroxyindoleacetic acid (5-HIAA), chromogranin A (CGA), gamma-aminobutyric acid (GABA), somatostatin (SST).

Introduction

The afferent innervation of the gastrointestinal (GI) tract consists of intrinsic and extrinsic sensory neurons that respond to nutrients, chemicals or mechanical stimuli within the gut lumen. Most stimuli do not interact directly with the afferent nerves but instead activate specialised cells in the epithelium in a process of sensory transduction. It is thought that one of the first steps in this process is the release of serotonin (5-HT) from the enterochromaffin (EC) cells (for reviews on the sensory transduction process, see Raybould, 2002; Bertrand, 2003; Blackshaw *et al.*, 2007; Gershon & Tack, 2007; Grundy, 2008). The EC cells are a sub-type of enteroendocrine (EE) cells which are found among the enterocytes of the intestinal epithelium. The EC cells are responsible for the production and storage of the largest pool of 5-HT in the body (Erspamer, 1954; Gershon & Tack, 2007). Released 5-HT can act on the intrinsic nerves (reviewed by Schemann, this issue) and vagal endings (reviewed by Zagorodnyuk *et al*, this issue). This review will focus on the role of 5-HT in sensory transduction and examine how the EC cell produces and releases 5-HT. We will explore recent developments that have helped to elucidate some of the proteins that allow EC cells to sense the luminal environment. Finally, we will highlight some of the findings from new studies using electrochemical techniques which allow the real-time recording of 5-HT concentrations near to the EC cell.

5-HT is an important paracrine signalling substance in the GI tract

Serotonergic transmission from the raphé nuclei acts on most brain areas while enteric neurons utilise 5-HT for some reflexes and synaptic potentials (e.g., Monro *et al.*, 2002;

Monro *et al*., 2005). It is, however, the non-neuronal EC cells that produce, store and release the largest pool of 5-HT in the body (Erspamer, 1954; Gershon & Tack, 2007). 5-HT released from the EC cells modulates a large number of GI reflexes in health and disease (for reviews see Furness *et al*., 1999; Kirkup *et al*., 2001; Bertrand, 2003; Grundy & Schemann, 2005). 5-HT and other EE cell mediators, such as cholecystokinin (CCK) or adenosine-5'-triphosphate (ATP), are thought to act in concert to initiate or maintain motor and secretory patterns tuned to the contents and to the region of intestine. For example, mechanical stimuli applied to the mucosal epithelium releases 5-HT into the lumen which enhances peristaltic reflexes (e.g., Bülbring & Crema, 1959; Kirchgessner *et al*., 1992; Foxx-Orenstein *et al*., 1996; Grider *et al*., 1996; Linden *et al*., 2003; O'Hara *et al*., 2004). Similarly, nutrients - such as short or long chain fatty acids, peptides, glucose - or chemical stimuli (e.g., acid, base) seem to act at the epithelial border by causing release of 5-HT from the EC cells or other sensory mediators from the EE cells (Raybould, 2002; Bertrand, 2003; Blackshaw *et al*., 2007; Gershon & Tack, 2007; Grundy, 2008).

<Figure 01>

The release of 5-HT from the EC cells is not always beneficial. A very high level of release is one of the main causes of symptoms such as the diarrhoea associated with cholera (Lundgren, 1998; Turvill *et al*., 2000), the nausea due to chemo- or radiation therapy (Sanger, 1990) or the flushing and heart palpitations associated with carcinoid tumours (McCormick, 2002). The central position of EC cells in these symptoms has led to the proposal that they contribute to similar problems associated with chronic intestinal diseases such as inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS).

This has led to a great deal of interest in the effects of inflammation on EC cells and the 5-HT they release (e.g., Lomax *et al.*, 2006; Mawe *et al.*, 2006; Keating *et al.*, 2008). Interestingly, it is not always clear from individual measures of 5-HT levels or EC cell numbers if the overall availability of 5-HT goes up or down in such cases. For example, in early studies of ulcerative colitis (UC) patients, the numbers of EC cells decreased (e.g., Verity *et al.*, 1962), while in later studies the numbers of EC cells increased (e.g., El-Salhy *et al.*, 1997). More recently, Coates *et al* (2004) have found that the number of EC cells decreased in patients with severe UC but found no change in mild UC or in tissues from irritable bowel syndrome (IBS) patients. In contrast, Dunlop *et al* found an increase in mucosal 5-HT turnover in post-infectious IBS (Dunlop *et al.*, 2005).

Animal models of inflamed bowel have also yielded conflicting results. In a trinitrobenzene sulfonic acid (TNBS) model of mouse colitis, Linden *et al* (2005a) showed increased 5-HT availability due to a decrease in serotonin reuptake transporter (SERT)-dependent 5-HT uptake with no change in EC cell numbers, while previous studies of TNBS models of guinea-pig colitis and ileitis found an increase in EC cell numbers (Linden *et al.*, 2003; O'Hara *et al.*, 2004). On the other hand, a consistent finding of these studies was that levels of SERT expression were decreased. The central role of SERT in controlling 5-HT availability is highlighted in a new study by Bischoff *et al* showing that rats lacking SERT have a much more severe TNBS induced colitis (Bischoff *et al.*, 2009).

Several recent studies have explored the immunological link between inflammation and 5-HT availability. Wang *et al* have shown that during parasitic infection a Th2-based mechanism causes an increase in the number of EC cells and the amount of 5-HT

(Wang *et al.*, 2007). In support of this, Motomura *et al* have shown that a Th2-mediated inflammation causes similar changes as compared to a Th1 mediated inflammatory response to the same parasitic infection (Motomura *et al.*, 2008). Taken together these studies show that disease state such as inflammation can strongly regulate 5-HT availability.

The production, release, uptake and degradation of 5-HT in the intestine

The EC cells produce 5-HT from the dietary amino acid L-tryptophan using the rate limiting enzyme tryptophan hydroxylase (TpH) (Verbeuren, 1989). In the gastrointestinal tract TpH isoform 1 has been localized to EC cells (Yu *et al.*, 1999) while the second isoform, TpH2, has been localized to enteric neurons and central raphé neurons (Walther *et al.*, 2003). TpH1 converts L-tryptophan to 5-hydroxytryptophan (5-HTP). The non-rate limiting enzyme L-amino acid decarboxylase (L-AADC), contained in EC cells, then converts 5-HTP to 5-HT (Hakanson *et al.*, 1970; Verbeuren, 1989). Newly produced 5-HT is packaged into granules/vesicles by the vesicular monoamine transporter 1 (VMAT1); this isoform is specific for EC cells and a small proportion of adrenal chromaffin cells (Rindi *et al.*, 2004; Schafermeyer *et al.*, 2004). 5-HT is released mainly from the granules stored near the basal border of the EC cell, but some studies have also identified granules near the apical membrane (Nilsson *et al.*, 1987) and demonstrated that 5-HT can be selectively released into the lumen (Ahlman *et al.*, 1981; Forsberg & Miller, 1982; Fujimiya *et al.*, 1998b) and found in the faeces (Fukumoto *et al.*, 2003; Tsukamoto *et al.*, 2007). What causes the release of 5-HT will be covered in detail in later sections of this review.

<Figure 02>

5-HT released from the granules stored near the basal border of the EC cell enters the lamina propria where it can interact with nerve terminals, immune cells and can be taken up into the blood by the platelets. The EC cells are in constant migration from the crypt to the tip of the villus (e.g., O'Hara & Sharkey, 2007); one consequence of this is that no close contacts with nerve terminals or immune cells can be maintained. Indeed close contacts between EC cells and other structures are not common in the first place (Wade & Westfall, 1985). Thus to affect the nerve terminals, 5-HT must be released in a paracrine manner. The high concentrations of 5-HT released from the EC cell can activate the intrinsic or extrinsic sensory nerve terminals via 5-HT_3 receptors (Hillsley *et al.*, 1998; Bertrand *et al.*, 2000), while at lower concentrations it activates 5-HT_4 or 5-HT_{1P} receptors (Grider *et al.*, 1996; Pan & Gershon, 2000). Many *in vitro* studies have shown that these receptors are important for the initiation or propagation of enteric reflexes such as peristalsis (Kadowaki *et al.*, 1996; Tuladhar *et al.*, 1997; Grider *et al.*, 1998; Jin *et al.*, 1999) or secretion (Sidhu & Cooke, 1995; Cooke *et al.*, 1997a, b).

The actions of 5-HT are terminated by uptake *via* the serotonin re-uptake transporter (SERT - a Na^+/Cl^- dependent transporter) into epithelial cells (Martel *et al.*, 2003). This is the same SERT protein that is found in platelets, enteric neurons and central raphé neurons (Gershon, 2004). SERT is the target for important therapeutic drugs such as fluoxetine and paroxetine, members of the serotonin-selective re-uptake inhibitor family. SERT has been localized to many intestinal epithelial cells using immunohistochemistry (Wade *et al.*, 1996) and northern blot analysis (Chen *et al.*, 1998), and appears to be

present in the apical and basal membranes of the epithelial cell (Gill *et al.*, 2008). The SERT gene is subject to a number of naturally occurring polymorphisms in the coding and promoter regions, some of which have been linked to the symptoms of Irritable Bowel Syndrome (IBS) (e.g., Yeo *et al.*, 2004) or to the pharmacogenomics of IBS treatment (Camilleri *et al.*, 2002; Scherl & Frissora, 2003). A novel splice variant of SERT has been identified that is specific for the intestinal epithelium (Linden *et al.*, 2005b; Linden *et al.*, 2009) but has yet to be linked to disease.

Once SERT has brought 5-HT into the epithelial cells it is degraded to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase A (MAO_A), an enzyme localised to all epithelial cells of the intestine (Egashira & Waddell, 1984; Rodriguez *et al.*, 2001). Alternatively, 5-HT released into the lamina propria may enter the portal circulation and be found either as free 5-HT or within platelets (*via* the actions of SERT). As the portal circulation is processed by the liver the free 5-HT is rapidly degraded by liver enzymes. About one third is degraded by MAO_A to 5-HIAA which is commonly detected in urine. The remaining two thirds of 5-HT is degraded to the metabolite 5-HT-*O*-glucuronide (Gershon *et al.*, 1989; Verbeuren, 1989). Importantly, 5-HT taken up by platelets is protected from degradation in the liver and enters the general circulation.

<Figure 03>

Co-storage of other bioactive compounds in EC cells

5-HT is co-stored in the EC cells with a variety of other paracrine/hormone substances.

EC cells contain the vesicle storage protein chromogranin A (CGA); CGA is also expressed in most of the other enteroendocrine cells (Facer *et al.*, 1985; Rindi *et al.*, 2004). Once VMAT1 has transported 5-HT into the vesicle, CGA is thought to bind to it allowing high concentrations of 5-HT to be stored inside the granules, as has been demonstrated for adrenaline in chromaffin cells (Montesinos *et al.*, 2008). The presence of CGA allows EC cells to be identified using a silver reaction, a process which is still in use today (Grimelius, 2004). Interestingly, CGA can also be released with 5-HT (Okumiya & Fujimiya, 1999) and may be cleaved into an active hormone form (Curry *et al.*, 2002), though the physiological relevance for this in the intestine has not been established. Of the additional substances contained in EC cells, perhaps most important is melatonin. 5-HT is a precursor for melatonin synthesis and many EC cells have been shown to contain melatonin (Bubenik, 2002) and to release it to cause physiological effects (Sjöblom & Flemström, 2003). Another interesting transmitter contained in EC cells is gamma-aminobutyric acid (GABA). Of the epithelial cell types, GABA is present only in the EC cells (Oomori *et al.*, 1992) and as such it has been used as a marker to help in the enrichment of EC cell cultures (Schafermeyer *et al.*, 2004). It also seems likely that ATP, or a related purine, is co-stored with 5-HT. Recently discovery of vesicular nucleotide transporter (Sawada *et al.*, 2008), which transports ATP into vesicles, should allow definitive studies on this matter.

There are a variety of other substances that are in EC cells but have an unclear physiological role or were originally thought to be in EC cells but were later shown not to be. The relatively newly discovered gut hormone uroguanylin is present in all EC cells (Perkins *et al.*, 1997) while the related peptide guanylin was originally proposed to be in EC cells (Cetin *et al.*, 1994), but further study showed this was due to the guanylin antibody cross-reacting with uroguanylin (Perkins *et al.*, 1997). EC cells have also been

shown to contain the opioid precursor dynorphin (Cetin, 1988). Motilin was proposed to be contained in EC cells (Pearse *et al.*, 1974) but later research suggests this is not the case (Forssmann *et al.*, 1976; Fujimiya *et al.*, 1998a). Substance P was initially reported to be in some EC cells (Heitz *et al.*, 1976), however, later studies either found only a small amount (Alumets *et al.*, 1977) or none (Sokolski & Lechago, 1984). The story of substance P is still unclear as it has again been reported to be released from EC cells (Simon *et al.*, 1992), and a posthumous review of the work of Vittorio Erspamer suggests that it is stored in the EC cells of some species and in some EC cell derived tumours (Severini *et al.*, 2002; Grundy, 2008).

EC cell transduction machinery

There are two ways that stimuli in the intestinal lumen may be detected by the afferent nerve terminals located in the lamina propria. First, they may be ferried across the epithelium by specific transport proteins, as are most nutrients once they are broken down. Once across, these stimuli could interact with specialised receptors on the nerve terminal. This is the mechanism hypothesised by Liu *et al* (1999) for glucose and it is clear that capsaicin directly activates afferents through TRPV1 receptors. Second, receptors and transduction machinery may exist on the luminal aspect of EE cells or other specialised cells within the mucosal epithelium. The sensory mediators these cells release are known to act on receptors on the afferent terminals (Blackshaw *et al.*, 2007). As alluded to previously in this review, this is the sensory transduction process which we believe most stimulants act through.

In support of this second mechanism, there is evidence for taste transduction machinery

on intestinal epithelial cells. The presence of the taste G-protein α-gustducin has recently been shown in some EE cells (Hass *et al.*, 2007; Sutherland *et al.*, 2007). Similarly, sub-sets of EE cells (including EC cells) have been shown to contain taste receptors (T1R and T2R), second messenger systems (PLCβ2) and channels (TRPM5) that form part of the taste transduction machinery normally associated with the tongue (Dyer *et al.*, 2005; Bezençon *et al.*, 2007; Sutherland *et al.*, 2007; Kidd *et al.*, 2008; Mace *et al.*, 2009; Young *et al.*, 2009). Recently, Nozawa *et al* (2009) have shown that TRPA1, the receptor for pungent compounds such as mustard, is also expressed by EC cells. A good review of this very active area of research is Rozengurt and Sternini (2007).

The evidence for functional transduction machinery of the other senses in the GI tract is not as convincing as that for taste. Hofer *et al* (1999) showed that the G-protein transducin, present in the eye and in some taste receptors, was also present in GI epithelium. More recently, Mace *et al* have confirmed this and have shown that transducin can couple to taste machinery (Mace *et al.*, 2009). A recent study by Braun *et al* has shown that some of the smell machinery is present in the gastrointestinal tract (Braun *et al.*, 2007). In particular, a subset of olfactory receptors was detected, a finding that was supported by Kidd *et al* who showed that activation of these receptors stimulated 5-HT release from EC cells (Kidd *et al.*, 2008).

Glucose sensing in the GI tract is apparently more complicated than for other nutrients. It has been linked to taste receptors (Mace *et al.*, 2009) but it has also been linked with the mechanisms of glucose sensing used by the pancreatic β-cells (Raybould, 2002; Raybould *et al.*, 2004). Thus far, K_{ATP} (Kirchgessner *et al.*, 1996), Na^+/glucose

co-transporter 1 (SGLT1) (Liu *et al.*, 1999; Kidd *et al.*, 2008) and glucose transporter 2 (GLUT2) (Mace *et al.*, 2007) have all been found to play roles. For a short review of the glucose sensing machinery in the GI tract, please see Dyer *et al* (2007).

5-HT release from the EC cell

Much of the mechanistic data on how EC cells work has come from Racké, Schwörer and colleagues who over the years have described 5-HT overflow from *in vitro* segments of intestine from a variety of small and large animals (for review, see Racké *et al.*, 1996). They found that 5-HT release is generally *via* an external Ca^{++}-dependent process utilising L-type calcium channels (Forsberg & Miller, 1983). However, upon muscarinic receptor activation 5-HT release can occur by utilising calcium from internal stores. Release of 5-HT can also be evoked by agonists at a variety of receptors such as adrenoceptors, muscarinic receptors or 5-HT_3 autoreceptors, while release can be inhibited by activation of $GABA_A$, nicotinic or somatostatin (SST) 2 receptors or 5-HT_4 autoreceptors (Gebauer *et al.*, 1993).

The 5-HT measured using these traditional overflow methods is the sum of the output of many EC cells measured many minutes after release has occurred and is far from the site of action; thus, temporal and spatial information is lost. It is not surprising then that other techniques have been used in an attempt to look at the activity of only a few EC cells at a time. Studies of calcium transients in small numbers of EC cells show clearly that apparently identical EC cells respond to transmitter or calcium channel agonist differently (Satoh *et al.*, 1995; Lomax *et al.*, 1999; Satoh *et al.*, 1999); although for the most part the pharmacological features found by Racké and Schwörer have been

supported.

Studying single EC cells *in vitro* has been difficult as they are a relatively small proportion of the epithelial cells, perhaps 1 - 3% in human (Bose *et al.*, 2000; Coates *et al.*, 2004). In addition, there are many other types of EE cells with similar structural properties (Wade & Westfall, 1985). A successful approach at enriching the EC cells has used techniques originally developed for the histamine containing EC-like cells of the stomach (Oh *et al.*, 2005). Using successive sucrose gradients, Schafermeyer *et al* were able to enrich the EC cell fraction (Schafermeyer *et al.*, 2004) and more recently this technique has been used by Doihara *et al* (2009). Kidd *et al* have succeeded in enriching the EC cells using fluorescence-activated cell sorting techniques to the point that gene chips could be used to show expression of some taste machinery mRNA (Kidd *et al.*, 2006; Modlin *et al.*, 2006; Kidd *et al.*, 2008). Finally, Braun *et al* have purified human EC cells isolated by laser micro-dissection (Braun *et al.*, 2007).

A further attempt to look at single cells has utilised an EC cell model, the BON cell, which is derived from a metastatic human carcinoid tumour of the pancreas (Evers *et al.*, 1994). BON cells have been used as a model of EC cell function (Christofi *et al.*, 2004; Tran *et al.*, 2004) and to investigate the release of 5-HT by D-glucose application or mechanical stimulation (Kim *et al.*, 2001a; Kim *et al.*, 2001b). Recent studies have questioned the value of the BON cell as an EC cell model (Siddique *et al.*, 2009) and have instead used a cell line derived from a human carcinoid tumour of the small intestine (KRJ-I) (Kidd *et al.*, 2008). Other models include a human pancreatic endocrine cell line (QGP-1) (Doihara *et al.*, 2009) and a rat pancreatic delta cell line (RIN14B) (Nozawa *et al.*, 2009). Finally, there is the potential for a promising new model of the EC cell from work being done using the 'immortoMouse' (Whitehead & Robinson,

2009).

Electrochemical detection of 5-HT release

Another successful approach to examining only a few EC cells at a time has been to use electrochemical techniques to record 5-HT levels near the EC cells in real time. These techniques have been commonly used to record, for example, adrenaline release from chromaffin cells (Chow *et al.*, 1992), dopamine release from the caudate nucleus, noradrenaline release from the thalamus (Gerhardt *et al.*, 1984) and 5-HT from the raphé nucleus (Daws *et al.*, 2005). When molecules of a transmitter such as 5-HT are oxidised the transfer of electrons can be detected and quantified. This provides a direct and accurate measure of the number of molecules (i.e., the concentration) at the electrode tip (e.g., see Benzekhroufa *et al.*, 2009). Generally carbon fibre electrodes have been used, but boron-doped diamond-coated platinum electrodes have also been used successfully (Bian *et al.*, 2007; Patel *et al.*, 2007).

In the GI tract electrochemical techniques have been used to record 5-HT release selectively and quantitatively. Steady state levels of 5-HT near the mucosal surface have been measured and mechanically evoked 5-HT release has been determined. The function of SERT and its contribution to the endogenous 5-HT signal can also be measured. Together, these data allow the availability of 5-HT in healthy GI tissues to be assessed in real time and compared with that in diseased tissues. As these electrochemical techniques are relatively new in the GI field it is worth reviewing some of the evidence supporting its utility in detecting 5-HT released from EC cells.

Is 5-HT detected selectively by electrochemical techniques?

Given the number of substances that are contained in EC cells, and in other EE cells, it is important to know that 5-HT can be detected selectively using electrochemical techniques. One way to determine if compounds other than 5-HT are present and will contribute to the oxidation current recorded is to use cyclic voltametric techniques. Cyclic voltammetry ramps the voltage at the electrode while recording the current. 5-HT starts to oxidise at a specific voltage (usually around +300 mV) and when the voltage ramp reaches this potential much of the 5-HT on the electrode surface will oxidise producing a clear peak in the oxidation current. Another way to ensure that 5-HT is detected selectively is to coat the electrode with a thin film of Nafion, an anionic exchange resin that repels anionic species such as ascorbic acid or the metabolite 5-HIAA, and attracts the cationic 5-HT.

<Figure 04>

In each species tested to date, it has been shown that 5-HT is the major peak at lower oxidation potentials (< +500 mV). 5-HT peaks have been observed from the ileal mucosa of guinea pig (Bertrand, 2004), rat (Bertrand *et al.*, 2008c) and mouse (Bertrand, 2006b) and the colon of mouse (Bertrand *et al.*, 2008e). The catechols or dopamine oxidise at a lower potential than 5-HT but have not been observed during recordings made near the mucosa. As the voltage is ramped to around +700 mV a second peak is often observed from the mucosa. It could be that the signal is due to melatonin which can also be released from the EC cells. In support of this, the second

peak mirrors the 5-HT time course including run down, and exogenous melatonin oxidises at the same potential as the second peak seen from the mucosa (Bertrand, 2004). Recent preliminary work has confirmed that melatonin is released during electrochemical experiments (Patel, 2008) and has shown that the level of endogenous melatonin at the mucosal surface is increased in aged mice (P. Bertrand, unpublished data). As melatonin treatment has been shown to improve age-related changes in gall-bladder (Gomez-Pinilla *et al.*, 2006), the role of endogenous melatonin in gastrointestinal function may be a ripe new area of research.

Can the concentration of 5-HT be determined near the EC cell?

The concentration of 5-HT near the mucosal surface can be calculated based on the linear relationship between oxidation current and 5-HT concentration, using the current produced by known concentrations of exogenous 5-HT to calibrate this relationship. It is worth highlighting the fact that the concentration of 5-HT detected depends on how close the electrode is to the site of release, the EC cell. The minimum concentrations that can be detected away from the mucosa are less the 100 nM. As the electrode is brought close to, or touching, the mucosal epithelium, 5-HT concentrations rise to over 1 μM. Although the signal to noise ratio is very good, there are several problems and criticisms inherent in actually touching the epithelial surface with the electrode (see Vanden Berghe, 2008). Other studies looking at adult and neonatal guinea pig ileum (Bian *et al.*, 2007; Patel *et al.*, 2007) and at BON cells (Braun *et al.*, 2007) have not routinely converted oxidation current to 5-HT concentration, so we can not directly compare with previous studies (e.g., Bertrand, 2004).

<Figure 05>

What information do electrochemical recordings give?

As this is a new technique in the GI tract, it is important to ask what electrochemical recordings can tell us about the role of serotonin. Thus far electrochemical studies have measured steady state levels of 5-HT at the mucosal surface, and measured the kinetics of evoked 5-HT release following mechanical stimulation of the EC cells. 5-HT levels have been measured near the mucosal surface from a variety of animals (Bertrand, 2004, 2006b; Bian *et al.*, 2007; Patel *et al.*, 2007; Bertrand *et al.*, 2008a; Bertrand *et al.*, 2008c) and human surgical specimens (Bertrand *et al.*, 2008b).

<Figure 06>

Steady state levels of 5-HT detected electrochemically reflect the amount of 5-HT that is trapped in the unstirred layer. This level of 5-HT represents a steady state between escape into the bulk solution (the lumen) and reuptake by SERT into the epithelial cells (more about SERT below). As an example, the steady state levels of 5-HT in mouse colon are about 2 μM, lower than that found in the mouse ileum (Bertrand, 2006b) or in the ileum of other species such as rat (Bertrand *et al.*, 2008c) or guinea-pig (Bertrand, 2004) and similar to known distributions of EC cells (Sjölund *et al.*, 1983).

Mechanical compression of the epithelium is a challenge to the EC cell and so the mechanosensory function of the EC cell can be tested. When a glass rod (Bian *et al.*, 2007) or the electrode itself (Bertrand *et al.*, 2008c) is used to stimulate the EC cells an increase in the levels of 5-HT are detected. In addition, a contraction of the circular or

longitudinal muscle is a mechanical stimulus for 5-HT release (Bertrand, 2006a). The peak concentration of mechanically evoked 5-HT release is usually 2-4 times that of the steady state levels. For example, peak levels of 5 HT released in mouse colon are approximately 7 μM in response to compression of the mucosa versus 2 μM at steady state (i.e., the steady state levels were approximately 30% of peak compression levels). In mouse ileum compression evoked 10 μM peak 5-HT while steady state levels were 6 μM (Bertrand, 2006b).

5-HT uptake by SERT can be detected electrochemically

The actions of 5-HT in the GI tract are terminated by uptake *via* the SERT which is located in most epithelial cells (e.g., Gill *et al.*, 2008). As noted above, the levels of 5-HT reflect a steady state between escape into the luminal solution and transport by SERT into the enterocytes. Using electrochemical techniques, it is possible to infer SERT function in real time from GI tissues. Blockade of SERT with a reuptake inhibitor such as fluoxetine can increase peak levels of compression-evoked 5-HT release and can prolong the decay time of these responses. Furthermore, there is an increase in steady state levels of 5-HT near the mucosa (Bian *et al.*, 2007; Bertrand *et al.*, 2008c) and an increase in the decay times of exogenously applied 5-HT (pressure ejected onto the surface of the mucosa) (Bertrand *et al.*, 2008c). In contrast, is seems that blockade of SERT does not increase the peak level of 5-HT detected following contraction-evoked release (Bertrand *et al.*, 2008c).

<Figure 07>

What can the electrochemical determination of 5-HT concentration tell us about receptor activation?

Electrochemical methods allow the accurate measure of the absolute concentrations of 5-HT near the mucosa and, by extension, near the afferent nerve terminals that control GI function and sensation (Keating *et al.*, 2008). The absolute concentration of 5-HT near the nerve terminal is important because this may substantially alter the activation or desensitization of serotonin receptors on afferent nerve terminals, or on the EC cells themselves. For example, the peak concentration of 5-HT measured from mouse colon was approximately 7 μM, high enough to activate the 5 HT_3 receptor (Bertrand *et al.*, 2008a; Bertrand *et al.*, 2008e). The ligand-gated 5-HT_3 receptor can only be activated by relatively high (> 1μM) concentrations of 5-HT and to be exposed to these high concentrations, the receptor must be close to the site of 5-HT release. Near the EC cell concentrations are high while far from the receptor concentrations are lower as a result of dilution and reuptake. However, these lower concentrations of 5-HT are not inactive as all other 5-HT receptors are G-protein coupled requiring much lower (> 5 nM) concentrations of 5-HT for activation. Thus, we can speculate that the actions of 5-HT through the clinically important 5-HT_4 receptor could be far from the site of 5-HT release.

Are electrochemical methods sensitive to changes in 5-HT availability during development or disease?

Recent studies have used electrochemical techniques to explore the availability of 5-HT in animal models of development and disease. Bian *et al* has shown that mechanically stimulated release of 5-HT is increased by blockade of SERT in adult but not neonatal guinea pig ileum (Bian *et al.*, 2007). This suggests that SERT levels are lower in

neonatal ileum, a finding that was supported with western blot analysis (Bian *et al.*, 2007). The levels of 5-HT may increase during aging. Preliminary data has shown a 50% increase in 5-HT release from aged mice (21 months) compared to young mice (3-5 months)(P. Bertrand unpublished observations). Similarly, electrochemical determination of 5-HT availability has been seen to increase in a mouse model of inflammation (Bertrand *et al.*, 2008a). During DSS-colitis, the levels of 5-HT detected by electrochemical methods were almost doubled that found in inflamed tissues.

It will be interesting to see how functional measures of 5-HT availability, such as the electrochemical methods described here, compare to anatomical or genetic analyses in a variety of disease states. Taken together, electrochemical determinations appear to be a useful adjunct to traditional methods of measuring 5-HT availability.

How important is 5-HT to GI tract function?

As this review is about 5-HT as a sensory mediator or paracrine substance it is fair to ask how important 5-HT is in the greater scheme of GI tract control. It has been known for years that experimentally reducing 5-HT content in the GI tissues does not cause as dramatic a reduction in enteric reflexes as one might predict. For example, Bülbring and Lin (1958) showed that 5-HT overflow dropped by 90% during sustained peristalsis but that fluid transport only dropped by 50%. Adding the precursor 5-HTP increased 5-HT output but actually decreased fluid transport (Bülbring & Lin, 1958). Similarly, depletion of EC cell 5-HT by parachlorophenylalanine (pCPA) (Weber, 1970) did not affect gastrointestinal transit of a charcoal meal (Pourgholami & Goshadrou, 1995). Further, genetically reducing 5-HT availability does not cause death or a lack of GI function (Lrp5

over expression, Yadav *et al.*, 2008), (knockout of TpH1, Walther *et al.*, 2003).

Putting aside the inherent dangers of using genetic models which allow developmental plasticity to occur, it is fair to say that a decrease in 5-HT availability does not cause severe long-term GI tract dysfunction. There are several reasons why this may be the case. First, as noted 5-HT is one of many GI hormones released from EE and EC cells. It is clear that these hormones have individual as well as overlapping functions; thus, they can act in concert and act as a backup. Second, the GI tract is well known for its ability to adapt and change to the environment. Unlike most organs of the body, the GI tract is subject to an ever-changing chemical environment. Many of the toxins now used to block synaptic or neuromuscular transmission could well have been ingested accidentally by past generations (*e.g.,* hyoscine/belladonna) and so the GI tract has developed to overcome the problem of ingested toxins. Thus, while too much 5-HT can be toxic (*e.g.,* following chemotherapy), it seems that with too little 5-HT life still goes on thanks to these alternative mechanisms. Does this mean measuring 5-HT levels is not important for understanding the physiology or pathophysiology of the GI tract? No, on the contrary it means that 5-HT is a useful marker for a variety of disease states. The system of enzymes and transporters that produce and package 5-HT, and the EC cells and their host of proteins which control the release of 5-HT, are part of a highly adaptable system.

Future directions

Research on serotonin and EC cells in the GI tract has gained momentum in the last 10 years. We believe that some of the most exciting developments relate to the isolation

and genetic analyses of EC cells, and to the evoked release of 5-HT by a wide variety of stimulants with subsequent detection using electrochemical techniques.

The enrichment of EC cells has now been accomplished by several groups using centrifugation, cell sorting and laser micro-dissection. In addition, several new cell lines have been characterised and are as good as, if not better than, the venerable BON cell line. The final step may be to generate a transgenic mouse with EC cell-specific expression of a fluorescent protein, as has been done with GLP containing L-cells (Reimann *et al.*, 2008) and CCK cells (Samuel *et al.*, 2008). The enrichment of EC cells has allowed a global survey of EC cell genes to be carried out (Kidd *et al.*, 2006). These studies have identified a host of potential sensory transduction components including receptors for taste and smell (Kidd *et al.*, 2008), and have opened up the potential for comparative studies with diseased tissue (Kidd *et al.*, 2007).

There is a need to re-examine what stimulants cause EC cells to release 5-HT. Previous studies have found many nutrients and other stimuli evoke 5-HT release (Racké *et al.*, 1996) but whether this was due to a direct effect on the EC cell is not clear. The genetic clues gleaned recently have directed the choice of potential stimulants to be tested while the enrichment of EC cells has provided a clear target upon which to test. Well known compounds such as mustard (Nozawa *et al.*, 2009) or caffeine (Kidd *et al.*, 2008), as well as odorants found in roses or raspberries (Braun *et al.*, 2007) reliably evoked 5-HT release. Perhaps a more important goal will be to translate these insights into useful information about the physiology or pathophysiology of the EC cell. In this respect, electrochemical techniques may be useful as they can provide a continuous readout of EC cell activity within whole tissues and during normal function. The challenge here will

be to embed these sensors in the GI tract chronically and to expand the types of sensory mediators that they can detect. In the end, the EC cell is only one of several sub-types of EE cell, each with an important role in paracrine signalling.

Conclusions

The GI tract senses the luminal contents and signals to the extrinsic and intrinsic nerves in the wall of the gut. The EC cell plays a key role in helping to transduce these signals by converting chemical, nutrient or mechanical stimuli into the release of 5-HT. All proteins for the production, uptake and degradation of 5-HT are known. However, many exciting new findings have highlighted the role 5-HT plays in disease and uncovered the ways in which 5-HT release is controlled. Proteins once thought specific for taste or other sensory transduction systems have been implicated in controlling 5-HT release from EC cells. New techniques have allowed the genetics of enriched EC cells to be studied and electrochemical techniques have provided new insight into the kinetics of 5-HT release.

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Figure Legends

Figure 1. Side view of the intestinal wall showing the different layers. Top, a diagram showing a section of intestine with two villi highlighted and shown in detail below. These layers are, from the top: the mucosal epithelium (EPI) which contains the enterocytes and the enteroendocrine (EE) cells - specialised epithelial cells that contain neuroactive substances located in secretory granules. Several types of EE cell are depicted (different colours) including the 5-HT containing enterochromaffin (EC) cell (depicted releasing 5-HT near to afferent nerve terminals into the underlying lamina propria). The submucosal plexus (SMP) is next, with the cell bodies of secretomotor, vasodilator, and a population of intrinsic sensory neurons. The circular muscle (CM), followed by the myenteric plexus (MP) which contains the cell bodies of motor neurons, interneurons and a population of intrinsic sensory neurons; and finally, the longitudinal muscle (LM).

Figure 2. The production of 5-HT by the enterochromaffin (EC) cell. A diagram of a single EC cell (centre) with surrounding epithelial cells. The beginning of the synthesis pathway for 5-HT is represented at the top of the cell where dietary tryptophan is converted to 5-hyroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase 1 (TpH1). 5-HTP is then converted to 5-HT by the enzyme L-amino acid decarboxylase (L-AADC). Newly produced 5-HT is packaged into granules/vesicles by the vesicular monoamine transporter 1 (VMAT1). 5-HT is released mainly from the granules stored near the basal border of the EC cell (depicted), but studies have also identified granules near the apical membrane where release may also take place. Once released, 5-HT can be transported into surrounding epithelial cells by the serotonin

reuptake transporter (SERT) and degraded to 5-HIAA by monoamine oxidase A (MAO_A; located in mitochondria).

Figure 3. The cycle of 5-HT from the GI tract to liver and general circulation. Once released by the enterochromaffin (EC) cell, there are several possible routes that 5-HT may take. 5-HT released into the lumen may escape into the bulk solution (where it can be detected in the faeces) or may be taken up by the epithelial cells via the serotonin reuptake transporter (SERT). 5-HT released into the lamina propria (where it can interact with nerve terminals) may also be taken up into the enterocytes by SERT or may enter the blood. 5-HT in the blood is present as free 5-HT or is taken up by platelets via SERT. The portal circulation is first processed by the liver before the blood enters the general circulation. Free 5-HT in the blood is rapidly degraded by MAO_A (to 5-HIAA) or by glucuronidases, while 5-HT in platelets is protected from degradation. Thus, under normal conditions, only 5-HT stored in platelets enters the general circulation.

Figure 4. 5-HT can be detected selectively in the presence of other transmitters. A-C are cyclic voltammetry traces taken with the carbon fibre electrode touching the base of an organ bath during equilibration of drugs. All traces were taken with a scan rate of 0.47 V/s and are from the same electrode - background has not been subtracted. The bath solution was flushed with fresh physiological saline between panels. The region of the ramp from -350 mV to +920 mV is shown. The scale in panel C applies to all traces and the vertical dotted lines denote the time/voltage at which the peak current was produced. **A.** Two traces taken with adrenaline (ADR - 40 μM) alone (upper) with a peak current at +200 mV, and (lower) adrenaline with the addition of 5-HT (0.5 μM) with a peak current at +360 mV. **B.** Melatonin (MEL - 120 μM) alone (upper) with a peak

current at +710 mV and with the addition of 5-HT (0.5 μM; lower) with a peak current at +380 mV. **C.** Ascorbic acid (AA - 250 μM) alone with a peak current at +200 mV (upper) and with the addition of 5-HT (0.5 μM; lower) with a peak current at +350 mV. Note that oxidation potentials will be offset when different styles of electrochemical electrodes are used (e.g., Bian *et al.*, 2007; Patel, 2008).

Figure 5. Effect of electrode position on 5-HT concentration in guinea pig ileum. A. Spontaneous contraction of the circular muscle (CM) and release of 5-HT from guinea pig ileum. In the top traces the electrode was held ~200 μm above the mucosal surface. Only threshold concentrations of 5-HT were detected (<100 nM). In the same preparation, when the electrode was lowered to within ~100 μm, 5-HT concentrations more than doubled. **B.** Stretch activated release of 5-HT with the electrode in contact with the mucosa yielded 10 μM 5-HT. In contrast, with the electrode 200 or 100 μm away, 5-HT concentrations fell to 200 to 100 nM (traces from A. re-scaled to match the scale in B.).

Figure 6. A comparison of 5-HT levels detected electrochemically near the intestinal surface of animal models and human. 'Peak' refers to the peak concentration of the compression-evoked 5-HT release. SS refers to the steady state or background levels of 5-HT detected near the surface of the intestinal epithelium. Broadly speaking, the levels of compression-evoked 5-HT release varied between 10 and 25 μM while steady state levels varied between 2 and 10 μM. From the left, tissues examined were: human sigmoid colon (H-SigC) (Bertrand *et al.*, 2008b); guinea pig ileum (GP-Ileum) (Bertrand, 2004, 2006a); mouse ileum (M-Ileum) (Bertrand, 2006b); mouse distal colon (M-DC) and mouse dextran sulphate sodium colitis (DSS M-DC)

(Bertrand *et al.*, 2008a); rat ileum (R-Ileum) and obese rat ileum (Fat R-Ileum) (Bertrand *et al.*, 2008d).

Figure 7. Overview of how the uptake of 5-HT may affect its actions. Left: serotonin re-uptake transporter (SERT) present in many intestinal epithelial cells. Right: enterochromaffin (EC) cell and endogenous 5-HT release. Bottom: nerve terminal from the intrinsic and extrinsic sensory nerves containing serotonin receptors, for example the 5-HT_3 receptor. 5-HT released from the EC cell might act directly on the sensory nerve terminals and then might be taken up by SERT. Alternatively, a large proportion of released 5-HT might be taken up directly by SERT before it has a chance to act on the sensory nerves. Evidence from recent electrochemical studies suggests that small amounts of endogenous 5-HT can act directly on the nerve terminal (5-HT_3 receptor depicted) before being taken up by SERT. However, 5-HT released in large amounts may, in part, be taken up by SERT before acting on the nerve terminals. A primary role of SERT may be to control the background levels of 5-HT. This is important because background levels of 5-HT control the balance between activation and desensitization of serotonin receptors.

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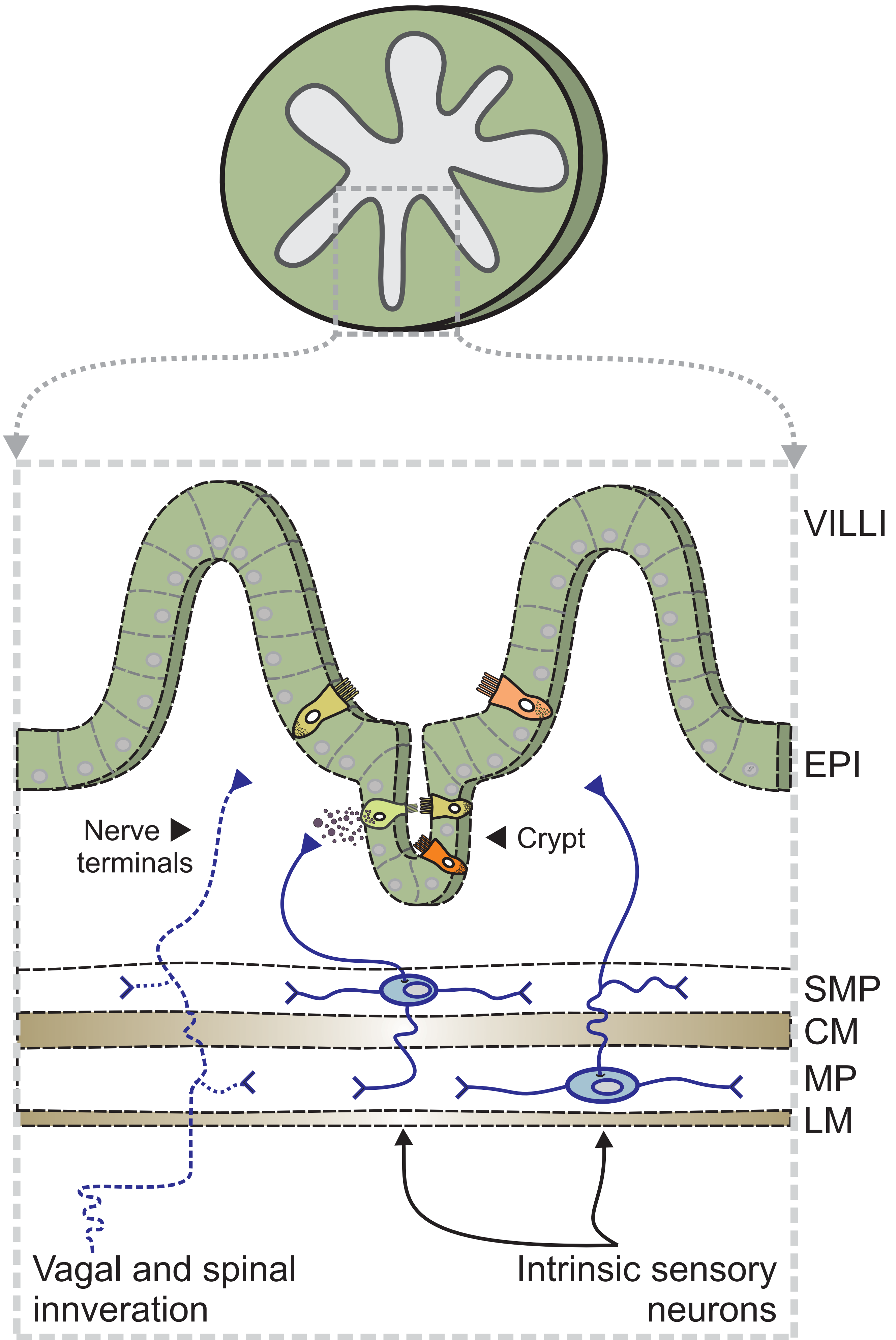
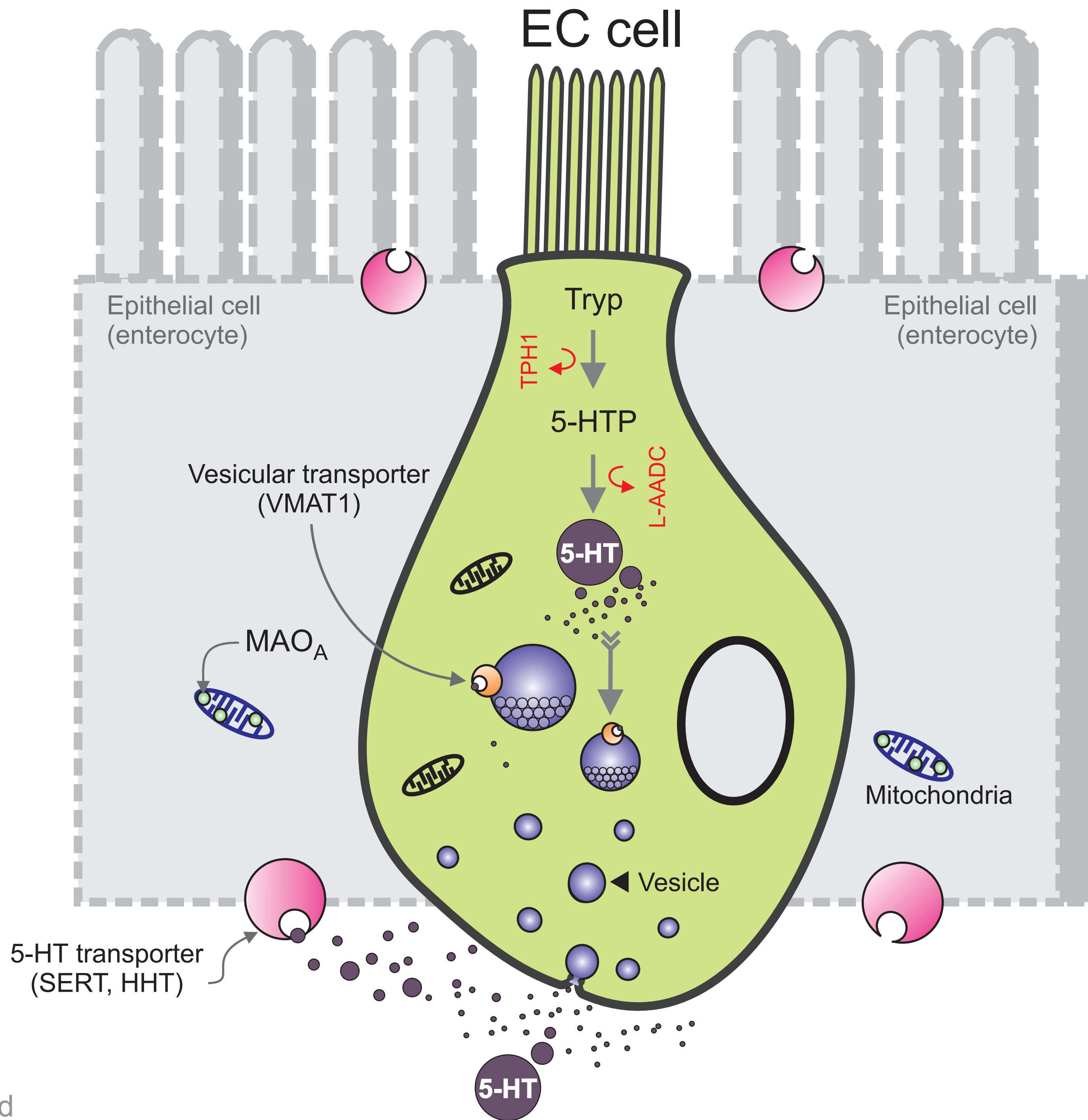


Figure 1, Bertrand



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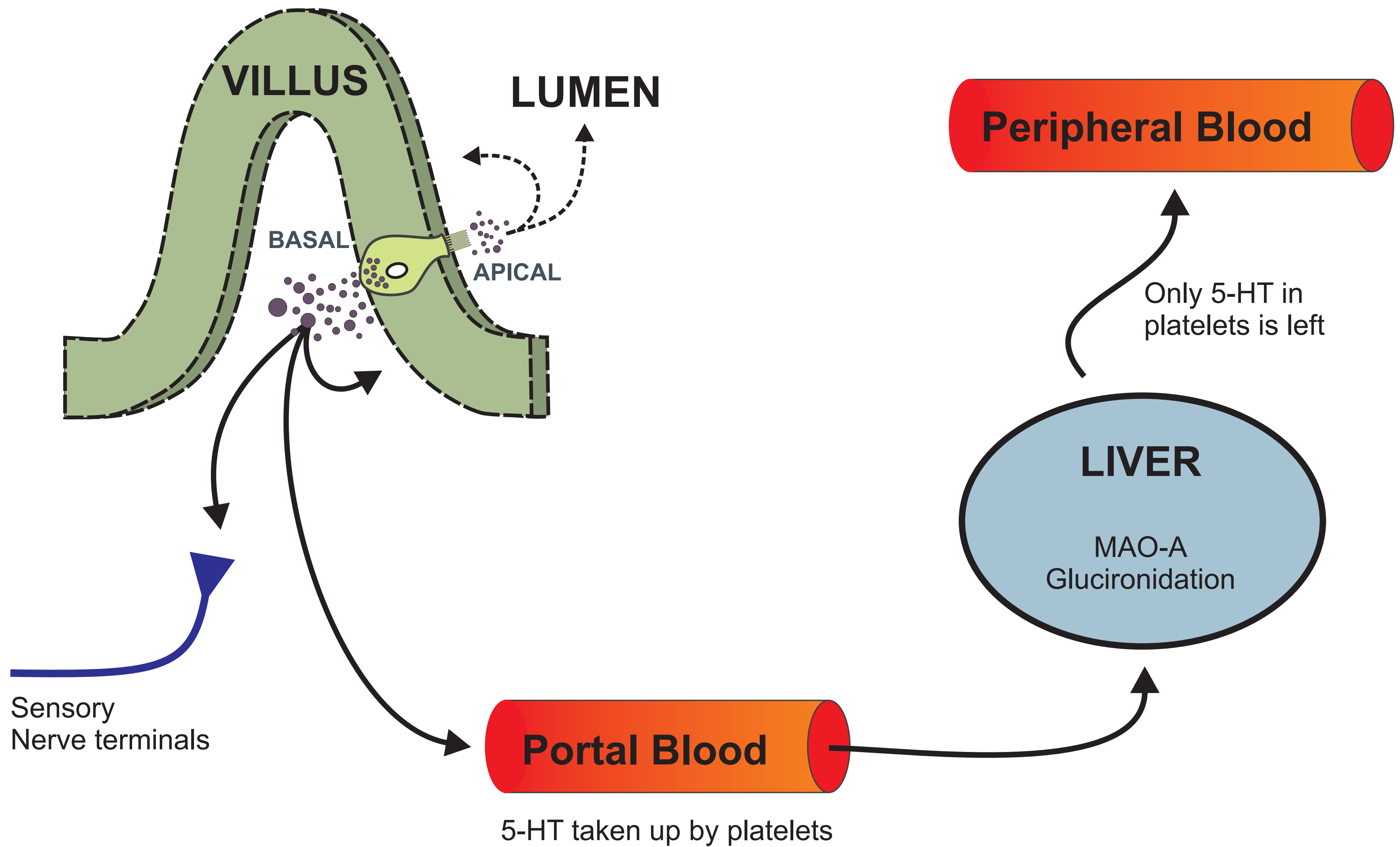

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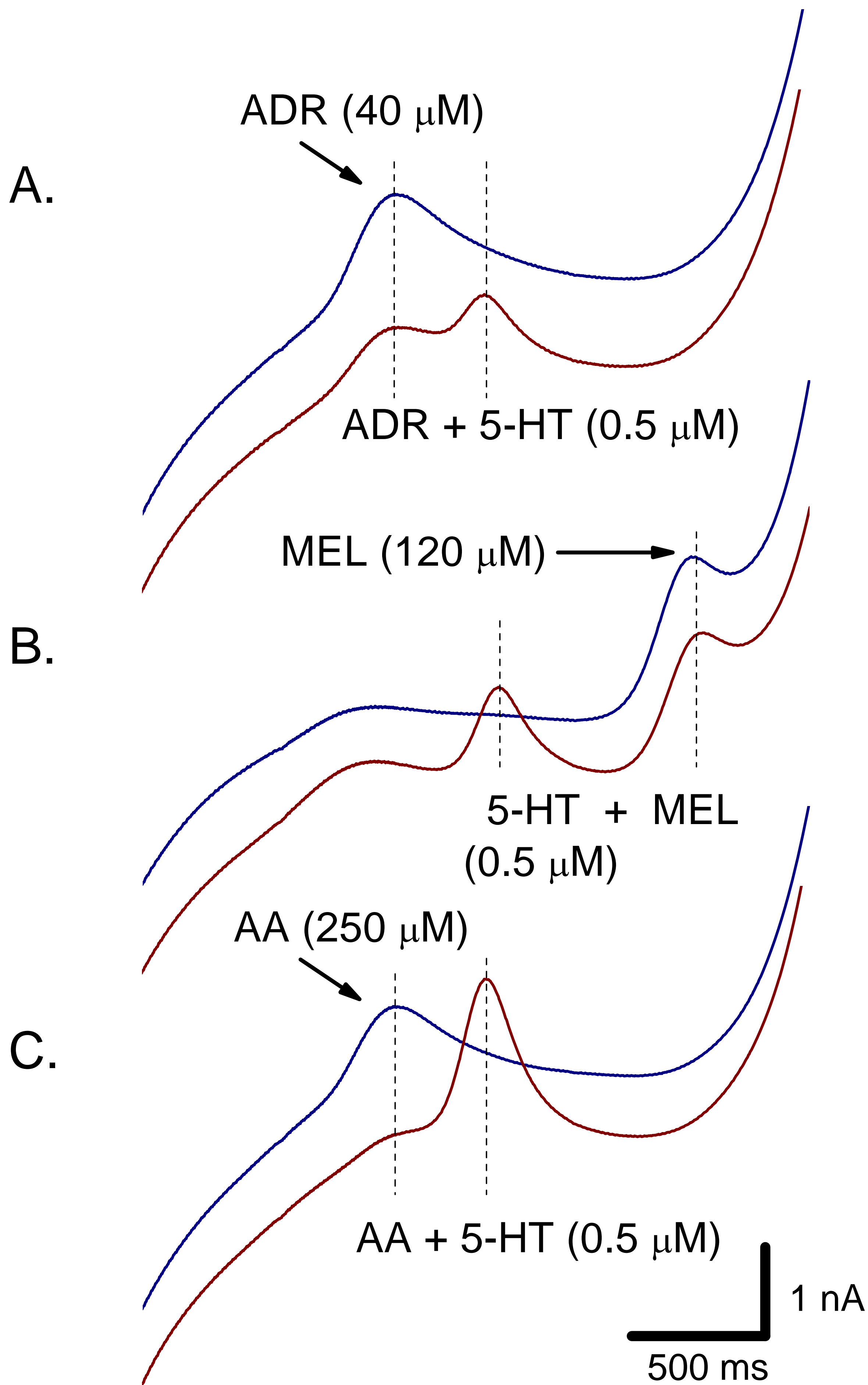

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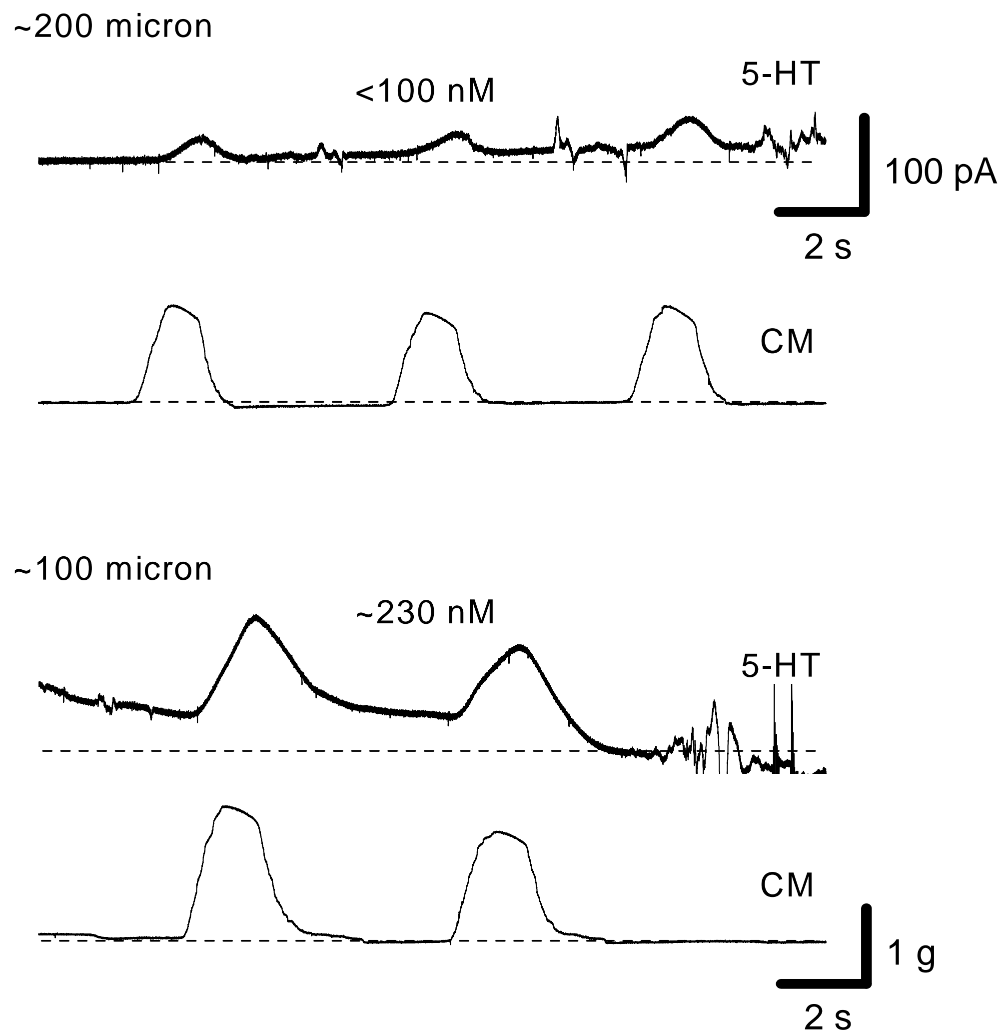

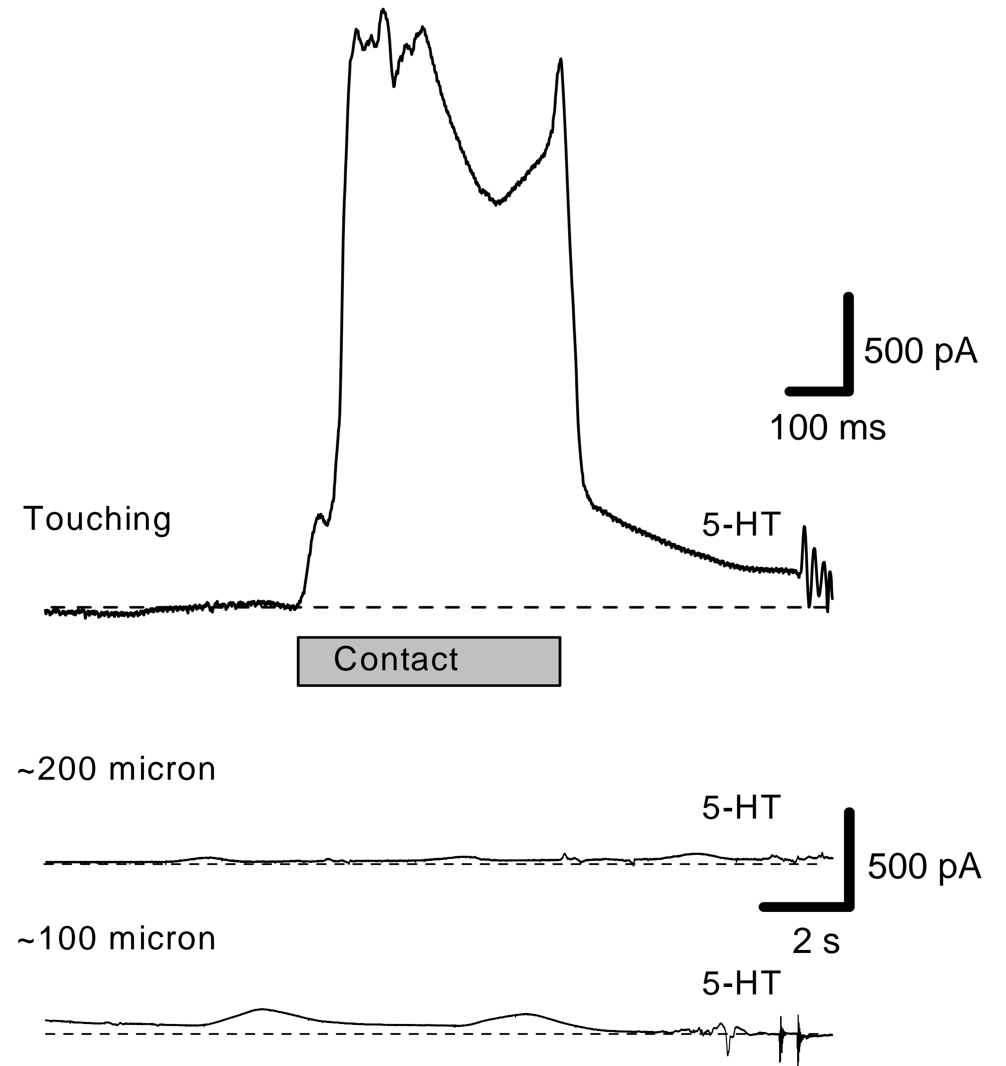

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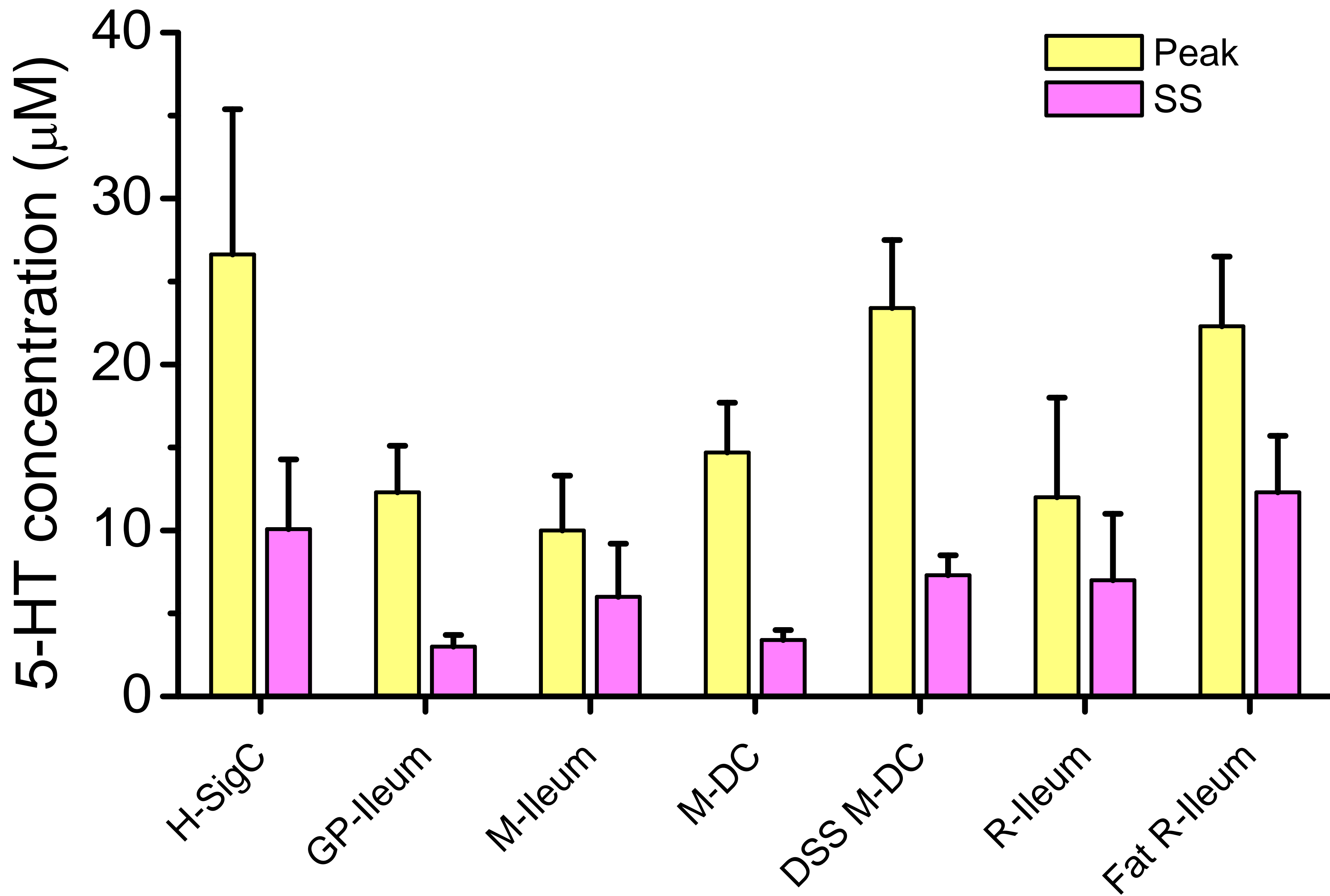

5-HT concentration (μM)
40
30
20
10
0
Peak
SS
H-SigC
GP-Ileum
M-Ileum
M-DC
DSS M-DC
R-Ileum
Fat R-Ileum

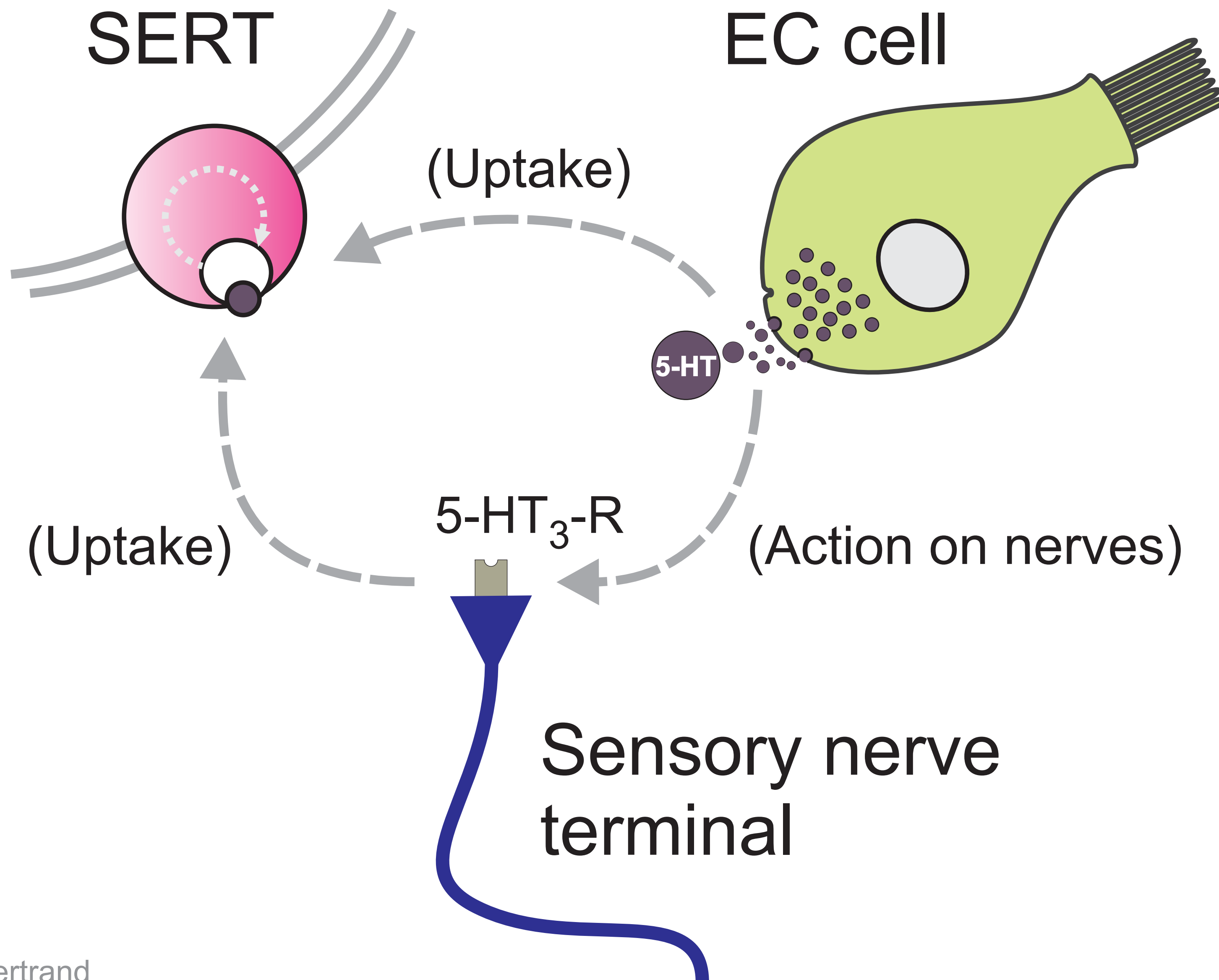

Figure 7, Bertrand