Glucagon like peptide 1 and peptide YY are in separate storage organelles in enteroendocrine cells

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Short Title: GLP-1 and PYY in L cells

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Abstract

A sub-group of enteroendocrine cells (L cells) release gastrointestinal hormones, GLP-1 and PYY, which have different but overlapping physiological effects, in response to intraluminal nutrients. Whilst their release profiles are not identical, how the plasma levels of these two hormones are differentially regulated is not well understood. We have investigated the possibility that GLP-1 and PYY are in separate storage vesicles. In this study, the subcellular location of GLP-1 and PYY storage organelles was investigated using double-labeling immunohistochemistry, super resolution microscopy and high resolution confocal microscopy. In all species tested, human, pig, rat, and mouse, most cytoplasmic stores that exhibited GLP-1 or PYY immunofluorescence were distinct from each other. The volume occupancy, determined, by 3-D analysis, overlapped by only about 10 ~ 20 %. At the lower resolution achieved by conventional confocal microscopy there was also evidence of GLP-1 and PYY being in separate storage compartments, but in subcellular regions where there were many storage vesicles, separate storage could not be resolved. The results indicate that different storage vesicles in L cells contain predominantly GLP-1 or predominantly PYY. Whether GLP-1 and PYY storage vesicles are selectively mobilised and their products are selectively released needs to be determined.

Keywords: L cells gastrointestinal hormones glucagon like peptide peptide YY super resolution microscopy

Introduction

Gastrointestinal enteroendocrine cells (EEC) that contain glucagon like peptide-1 (GLP-1) and peptide YY (PYY) (L cells) have key roles in the regulation of food intake and digestion. They detect luminal nutrients and the products of nutrient breakdown, including monosaccharides, free fatty acids, other metabolites of fat digestion, amino acids and bile salts, through specific receptors. In response to luminal nutrient stimuli they release endocrine hormones that are derived from two precursor proteins, proglucagon and the PYY precursor protein, preproPYY (Conlon 2002; Holst 2007). Proglucagon is processed by cell and tissue specific pathways to produce different final products or combinations of final products. In L cells, the products of proglucagon are GLP-1, GLP-2 and oxyntomodulin. The product of preproPYY is PYY, which is metabolised extracellularly to the biologically active product PYY3-36 (Grandt et al. 1992; Mentlein 1999). An overlapping population of EEC is cells containing both the typical K cell hormone, glucagon-like

insulinotropic hormone (GIP) and GLP-1. These K/L cells are common in the proximal intestine of rats, pigs and humans (Mortensen et al. 2003).

The different products of L cells have different, but overlapping, physiological effects. Major roles of GLP-1 are as an inhibitor of gastric emptying and as an incretin – it potently slows gastric emptying and increases secretion of insulin (Nauck et al. 1997; Holst 2007). Agonists of the GLP-1 receptor on pancreatic islets or the inhibitor of the GLP-1 degrading enzyme, dipeptidyl peptidase-IV (DPPIV), are used to treat diabetes (Kumar 2012; Russell-Jones et al. 2012). By contrast, PYY has no significant effect on basal insulin secretion and rather reduces glucose induced insulin secretion (Szecowka et al. 1983; Böttcher et al. 1989). PYY is a satiety factor, an inhibitor of gastric emptying and an inhibitor of intestinal fluid secretion (Cox 2007). GLP-1 mimics PYY in being a satiety factor and in gastric inhibition (Holst 2007) but it has an opposite effect on electrolyte secretion (Joshi et al. 2013). There is recent evidence that enteric neurons express the GLP-1 receptor and that GLP-1 excites these neurons (Richards et al. 2014). Enteric neurons are also excited by PYY (Cox and Tough 2002). Because GLP-1 and PYY have different, although overlapping, physiological roles, it might be thought that the conditions leading to their release may differ.

Recent FACS and immunohistochemical analysis has shown that subsets of EEC contain different relative amounts of GLP-1 and PYY (Brubaker 2012; Habib et al. 2012) and differential release could arise from the selective expression of receptors for particular nutrients by subsets of EEC. Another possibility is that GLP-1 and PYY are contained in separate storage organelles in the same EEC, and that these organelles can be selectively mobilised by different stimuli. This possibility is suggested by recent observations that ghrelin and nesfatin-1 are contained in separate storage organelles in gastric EEC (Stengel et al. 2010; Stengel and Tache 2012). In contrast to ghrelin and nesfatin-1, ultrastructural studies suggest that GLP-1 and PYY are contained in the same storage vesicles (Böttcher et al. 1984; Nilsson et al. 1991). This is supported by recent fluorescence histochemistry observations (Habib et al. 2013). However, the storage vesicles are around 200 nm in diameter, and may not be adequately resolved by fluorescence histochemistry. In one study, 15% of secretory granules were labeled only by PYY antibody indicating that storage vesicle population are heterogeneous (Nilsson et al. 1991). In the current work, we have investigated the colocalisation of GLP-1 and PYY in EEC from mouse, rat, pig and human intestine using super resolution microscopy and high resolution confocal microscopy.

Materials and methods

Tissue sources

Distal ileum and distal colon were obtained from 5 C57BL/6 mice (male, 6-8 weeks, 25-30 g), 2 Sprague-Dawley rats (male, 250-300 g) and 3 pigs (Large White strain; 1 male, 2 females, 90-100 kg). Mice and rat were from the animal facility of the Department of Anatomy and Neuroscience and pigs were from the Melbourne University School of Land and Environment. Mice were anaesthetized with isoflurane, and rats and pigs were rendered insensible with carbon dioxide, before tissues were taken. The human tissue samples (n = 2 patients, male 38 years and female 86 years, not obese) were jejunum that was discarded when a Whipple's procedure was applied to remove cancerous or diseased pancreas. Adjacent tissue samples were examined histologically and no intestinal tissue pathology was detected. Human jejunum was obtained from the Melbourne University Department of Surgery at the Austin Hospital (Austin Health). All procedures were conducted according to the National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee and the Human Research Ethics Committee (Austin Health). In all cases, tissues were obtained in the morning.

Immunofluorescence

The intestine was removed and placed in fixative (4% paraformaldehyde) overnight at 4°C. The next day the tissue samples were trimmed and washed 3x in phosphate buffered saline (PBS: 0.15M NaCl in 0.01M sodium phosphate buffer, pH 7.2). The tissue was then placed in PBS-sucrose-azide (PBS containing 0.1% sodium azide and 30% sucrose as a cryoprotectant) and stored at 4^{o}C overnight. The following day, tissue was transferred to a mixture of PBS-sucrose-azide and OCT compound (Tissue Tek, Elkhart, IN, USA) in a ratio of 1:1 for a further 24 h before being embedded in 100% OCT. Sections of 6 μm thickness were cut and mounted on coverslips (Menzel-Glaser #1.5, Thermo Fisher, Scoresby, Vic, Australia) that were coated with poly-L-lysine (P8920, Sigma-Aldrich, Sydney, Australia). Sections were air dried for 1 h and incubated with 10% normal horse serum for 30 min. Sections were then incubated with chicken anti-PYY (1:500 for human, pig and rat, 1:1000 for mice, GW22771, Sigma-Aldrich, Sydney, Australia), rabbit anti-GLP-1 (1:2000, #8912, from Center for Ulcer Research and Education, University of California, Los Angeles, courtesy Dr Helen Wong) at 4°C, overnight. Sections were washed for 3x 10 min in PBS prior to secondary antibody incubation with donkey anti-chicken DyLight™ 649 (Jackson Laboratories, West Grove, PA, USA) and with donkey anti-rabbit conjugated either with Alexa Fluor® 488 or 568 (Molecular Probes, Mulgrave, Vic, Australia) for 1 h at room temperature. Preparations were further washed (3x10 min) in PBS following secondary antibody incubation. Before being mounted, sections were briefly exposed to the nuclear stain, 4',6-diamidino-2-

phenylindole (DAPI), 1:5000, for 1 min. Glycerol was used as a non-hardening mountant to prevent compression of the sample. The anti- PYY antibody was raised against recombinant PYY (amino acids 29-40) and has been previously shown to reveal PYY containing enteroendocrine cells (Karaki et al. 2008). The anti-GLP-1 antibody was raised against synthetic human GLP-1(7-36). The immunoreactivity observed with the GLP-1 antibody was completely abolished by pre-incubating the diluted primary antibody with 3 x 10^{-7} M synthetic GLP-1, 7-36 (Phoenix Pharmaceuticals Inc, Burlingame, CA, USA) at 4 ^{o}C, overnight.

Microscopy

Fluorescence was visualised by super-resolution microscopy using the OMX Blaze-SIM (Structured Illumination Microscopy) system (Applied Precision, Issaquah, Washington State, USA) and by high resolution confocal microscopy (Zeiss Meta510 laser scanning confocal microscope, Carl Zeiss, Sydney Australia). For OMX Blaze-SIM, images were taken using a 60x objective lens. Immersion oil with refractive index between 1.512 and 1.516 was used to match with secondary antibody fluorophores. On every experiment day, a reference image using Tetraspeck fluorescent beads (Life Technologies, Melbourne, Australia) was taken in each channel and used to calibrate the system, correcting for position shift.

With confocal microscopy, images were taken using a 63x oil immersion lens with NA=1.4. Images were zoomed in 2~3 times further digitally and obtained at 1024 x 1024 pixel images. Z series images were taken at 1 µm optical thickness with 0.5 µm interval for 3 dimensional image analysis.

Image analysis

Image analysis was conducted using Imaris (Bitplane AG, Zurich, Switzerland) software. The 3D structures of fluorescence organelles was rendered using two different detection methods, spot detection and surface detection. Spot detection was used to measure the diameter of a sphere that was fitted to the particle. Surface detection was used to create a 3D structure based on the fluorescence intensity. The threshold was manually determined as the brightest background for each channel and applied to all images identically. The 3D volume of immunofluorescence was calculated from the sum of positive voxels above threshold. The amount of 3D-colocalization was calculated by dividing the overlapped volume by the total sum of volume of the two channels. The overlapping volume between two immunoreactive organelles was calculated by superimposition. Colocalisation of autofluorescent beads (reference image) was also measured and used as a normalisation factor.

Results

In intestinal samples from mice, rat, pig and human, enteroendocrine cells that were imaged by 3D-Structured Illumination Microscopy (3D-SIM) in double-labeled tissues contained organelles that were immunoreactive for only GLP-1 or only PYY, and less commonly for both peptides (Fig 1). In the majority of cases, organelles that were immunoreactive for GLP-1 or PYY showed no detectable immunoreactivity for the other peptide hormone. Storage organelles with centres 100 nm apart were resolvable.

The organelles were approximately spherical and 90 - 440 nm in diameter. The mean diameters were 150 – 170 nm and there was no significant difference between species or organelle immunoreactivity (Table 1). Some of the organelles were elongated or were closely aligned to form a donut like structure (Fig 1b′). Donut-like structures were commonly observed in human and pig but rarely in rat and mouse.

Images that were rendered in 3-D (Fig 2) were used to determine the overlap between GLP-1 and PYY immunoreactivity. Calculation of volume overlap was 8-10 % in pig and mouse colon, and about 19-22 % in human jejunum and rat colon (Table 1). There were some regions within cells where one type of storage vesicle, either GLP-1 or PYY, predominated (Fig. 1).

When double labeled cells were investigated at high magnification by confocal microscopy, separate storage could be partly resolved where the storage organelles were sparse in the cells (green and red arrow, Fig 3). However it was not possible to reliably distinguish whether the immunoreactivity was localised to the same organelles or whether different organelles were too close to be resolved. The immunoreactive organelles tended to be crowded in the basal parts of the cells and appeared as merged objects (Fig. 3). The apparent diameters of immunoreactive organelles in enteroendocrine cells from mouse, when observed by conventional confocal microscopy, were 400 ± 30 nm (GLP-1) and 430 ± 10 nm (PYY), compared to super-resolution values of 160 ± 10 nm for GLP-1 containing organelles and 150 ± 10 nm for PYY containing organelles (P<0.0001, unpaired t-test; Table 1).

Discussion

The present study demonstrates that the majority of storage organelles that contain detectable GLP-1 and PYY are separate in L cells of mouse, rat, pig and human. We were able to make this distinction using super-resolution (3D-SIM) microscopy. By electron microscopy, the storage organelles can be recognised as vesicles, that contain a dense core within which the peptide is contained, surrounded by a clear area and a limiting membrane (Varndell et al. 1985; Sundler et al.

1989). Using 3D-SIM, we showed that the immunoreactive cores were, on average, 150-170 nm in diameter. This is comparable to averages of 190 nm for the storage granules obtained by electron microscopy (Böttcher et al. 1986). The overall vesicle size is 200-300 nm, but the peptide containing core can be eccentrically located, up against the vesicle membrane (Varndell et al. 1985; Sundler et al. 1989). Thus super-resolution microscopy provides an adequate resolution to distinguish the secretory vesicles of L enteroendocrine cells. On the other hand, by conventional confocal microscopy the vesicles have apparent diameters of 400 to 450 nm, on average, and thus vesicles (or vesicle cores) that are close, but separate, will not be resolved. So it was only in regions where vesicles are sparse (often the apical ends) that separate vesicles could be distinguished using conventional confocal microscopy. Visual inspection identified some overlap of fluorescence and calculations of volume overlaps using Imaris software indicated colocalisations of about 10 % in pig and mouse, and about 20 % in human and rat (Table 1). These are possibly overestimates due to a failure to completely resolve apposed vesicles, for example when the hormone containing granular cores are close to the limiting membranes of adjacent vesicles. On the other hand, 80-90 % of storage vesicles contained only GLP-1 or only PYY in amounts that were above threshold for immunohistochemical detection. It is possible that vesicles that appear negative for one or other peptide actually contain amounts too low to be detected by fluorescence immunohistochemistry. Thus the majority of vesicles can be concluded to contain primarily one or other hormone.

In EEC from human and pig we observed storage vesicles that were arranged in ring or donut-like structures. This type of arrangement of secretory vesicles has been reported before, in the calyx of Held (Wimmer et al. 2006). In the calyx, the vesicles are arrayed around mitochondria, and it has been suggested that the mitochondria may be a source of ATP for transporters of the storage vesicle membrane (Wimmer et al. 2006).

The results raise the possibility that GLP-1 and PYY could be selectively released from an individual EEC. Although we are unaware of evidence of selective release of hormones from individual endocrine cells, neurons commonly contain more than one neurotransmitter and in some cases selective release of transmitters has been demonstrated (Adams and O'Shea 1983; Lundberg et al. 1989; Vilim et al. 1996). As an example, noradrenaline and neuropeptide Y are co-transmitters in neurons innervating the spleen; at low frequencies of stimulation release of noradrenaline is favoured, whereas at high frequencies the dominant transmitter is neuropeptide Y. The release of enteroendocrine hormones, like the release of neurotransmitters, is dependent on phasic increases in cytoplasmic free calcium (Reimann et al. 2006). In nerve endings, calcium transients can occur in microdomains that are related to sources of calcium from intracellular stores (Galante and Marty

2003) or to the locations of channels through which extracellular calcium enters (Neher 2006). Thus release of a transmitter or hormone can, at least in theory, depend on the proximity of its vesicular store to particular stores or ion channel clusters. Ion channels and stores through which transient microdomains of elevated calcium are generated can in turn be related to clusters of cell surface receptors (Berridge 2006). We have noticed sub-cellular regions in which one type of storage vesicle (GLP-1 or PYY) dominates. These may represent microdomains from which selective release could occur. Thus in the case of the L-cell it is feasible that receptors through whose activation GLP-1 is released have a special relation with the cellular machinery that controls GLP-1 secretion, and that a similar receptor and release machinery exists for PYY.

There is in fact evidence of differential release of GLP-1 and PYY. Infusion of glucose or peptone into loops of rat ileum caused similar increases in GLP-1 and PYY in the venous drainage, whereas taurocholate caused about 3 fold more PYY than GLP-1 release (Dumoulin et al. 1998). Intraluminal glucose infused into rat colon released both GLP-1 and PYY, whereas oleic acid and short chain fatty acids, butyrate, released PYY, but did not induce a significant release of GLP-1 (Plaisancié et al. 1995; Plaisancié et al. 1996; Anini et al. 1999). In human volunteers, intraduodenal infusion of a glucose load (3 kcal/min) caused a 4-fold increase in plasma GLP-1, but did not increase plasma PYY (Gerspach et al. 2011). Similarly, a dose of intragastric glucose that increased GLP-1 secretion more than 10 fold, increased PYY about 2 fold in human volunteers (Steinert and Beglinger 2011). Although differential release from the same enteroendocrine cells may contribute to the differences in the ratios of GLP-1 to PYY in the circulation in response to different stimuli, differences between cell populations may also contribute. There is emerging evidence that enteroendocrine cells that had been classified as single classes, notably K and L cells, are in fact not uniform (Brubaker 2012; Habib et al. 2012). Thus there are cells that contain GLP-1 with little or no PYY, and possibly cells that contain PYY but with little or no GLP-1. Moreover, there are significant numbers of cells (K/L cells) in the proximal small intestines of humans, pigs and rats that contain both incretins, GIP and GLP-1 (Mortensen et al. 2003).

In conclusion, 3D-SIM microscopy and immunofluorescence double labeling show that most hormone storage vesicles in L cells of human, mouse, rat and pig are immunoreactive for GLP-1 or PYY, but not for both peptide hormones. The implications of this finding for possible selective control of endocrine hormone release are not yet clear.

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Figure Descriptions

Fig. 1 3D-Structured Illumination Microscopy images of double-labeled enteroendocrine cells from human jejunum (**a**), pig (**b**), rat (**c**) and mouse (**d**) colon. Areas marked by the squares in the top panels are enlarged in the bottom panels (**a′-d′**). Individual storage vesicles that are immunoreactive only for GLP-1 (*green arrows*), only PYY (*magenta arrows*), or both (*yellow arrows*) are indicated. *Asterisks* indicate vesicles that are resolved by super-resolution, but which would be unresolved by conventional confocal microscopy. The *white arrowhead* in **b′** indicates storage organelles that are arranged in a donut-like structure. *Scale bars*: **a**-**d**, 1 µm; **a′-d′**, 200 nm.

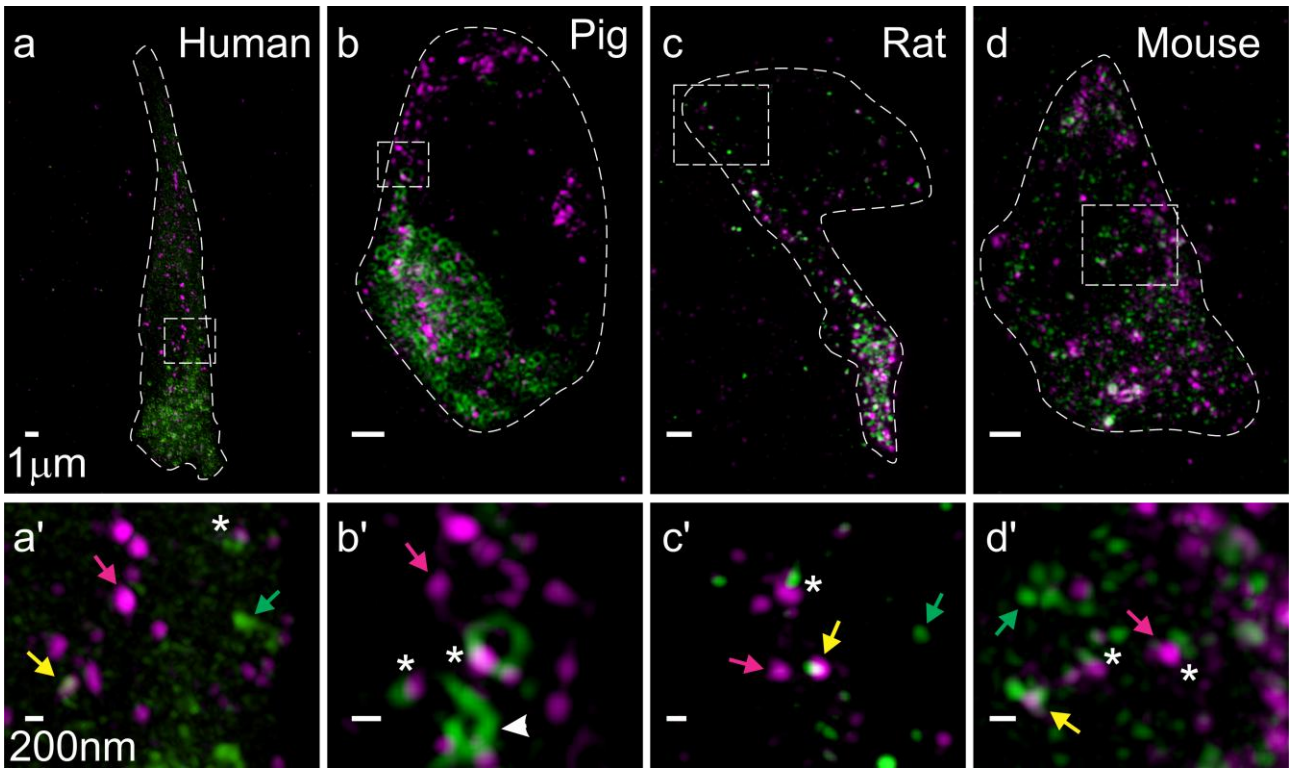
Fig. 2 Illustration of the overlap of localisation of GLP-1 and PYY within double-labeled enteroendocrine cells. Images from mouse colon. Upper row (**a**-**d**) shows raw images obtained using 3D-SIM imaging. Lower row (**a'-d'**) shows the same images following volume rendering using Imaris software. **a, a'**: GLP-1 localisation. **b, b'**: PYY localisation. **c, c'**: Organelles with immunoreactivity for both peptide hormones obtained by image subtraction. **d, d'**: Merged images showing both GLP-1 and PYY immunoreactivity. *Scale bar* in **a** applies to all panels.

Fig. 3 Conventional confocal microscopy image of an enteroendocrine cell from mouse distal ileum double labeled with GLP-1 (**a**, *green*) and PYY (**b**, *magenta*). **c**. Merged image, nucleus is stained with DAPI (*blue*). Z series images were taken at 0.5 µm optical thickness. The complete outline of the cell is shown by the white dotted lines in the upper panels. The hormone storage vesicles were fewer on the apical side (top of the cell) and were densely packed on the basal side (below the nucleus). Areas marked by the squares in the upper panels are enlarged in the bottom panels (**a'-c'**). Note that the image sizes of the organelles are approximately 200- 500 nm, which is larger than image sizes with 3D-SIM microscopy. In the majority of cases, the organelle images had immunoreactivity for both hormones (*asterisks*). Very few were immunoreactive for only one (*arrows*). *Scale bars*: 5µm for **a**-**c**; 1µm for **a'-c'.**

Table 1. Properties of hormone storage organelles in different species.

Species, Imaging mode (Number of cells assessed)	**Hormone**	**Organelle Diameter (nm)**		**Overlap (%)**	
		Mean	SEM	Mean	SEM
Human jejunum 3D-SIM (5)	GLP-1	160	10	21.6	7.1
	PYY	170	10		
Pig colon 3D-SIM (8)	GLP-1	150	10	10.3	2.3
	PYY	150	10		
Rat colon 3D-SIM (4)	GLP-1	150	10	19.1	10.9
	PYY	170	10		
Mouse colon 3D-SIM (7)	GLP-1	160	10	8.2	2.0
	PYY	150	10		
Mouse colon Confocal (7)	GLP-1	400*	30	N/A	N/A
	PYY	430*	10		

*= $P<0.0001$, comparing image sizes of organelles observed with 3-D SIM imaging and high resolution confocal microscopy in mouse enteroendocrine cells.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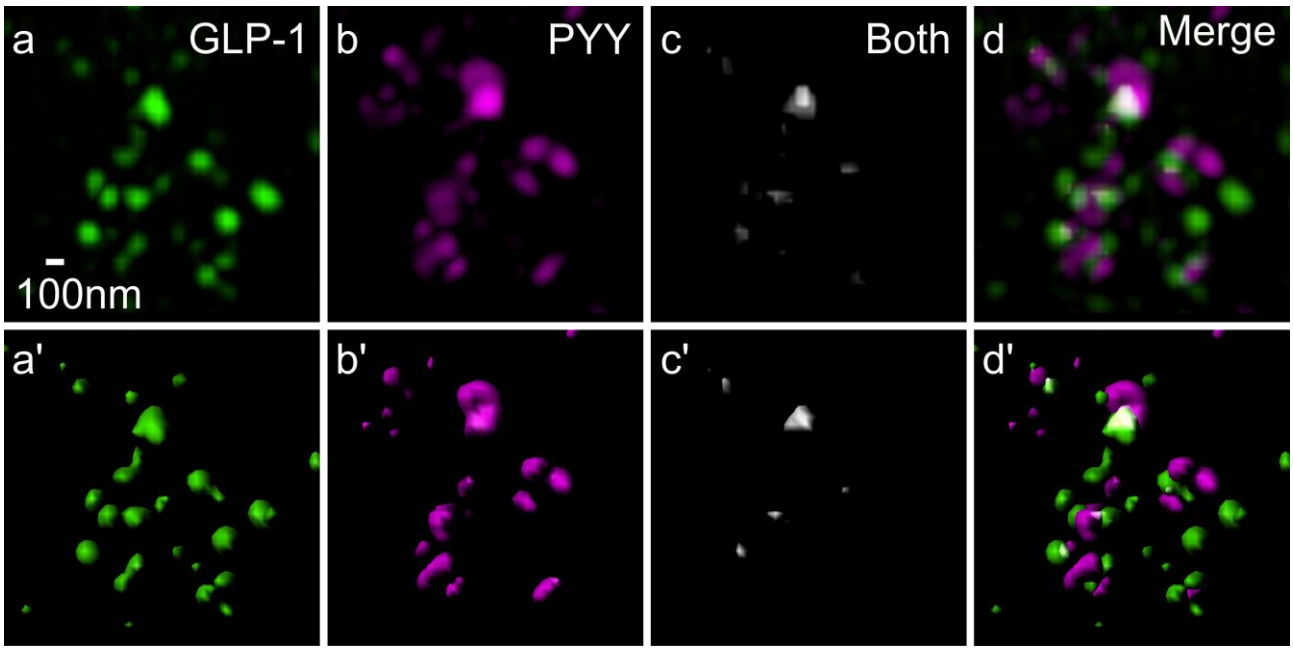
a
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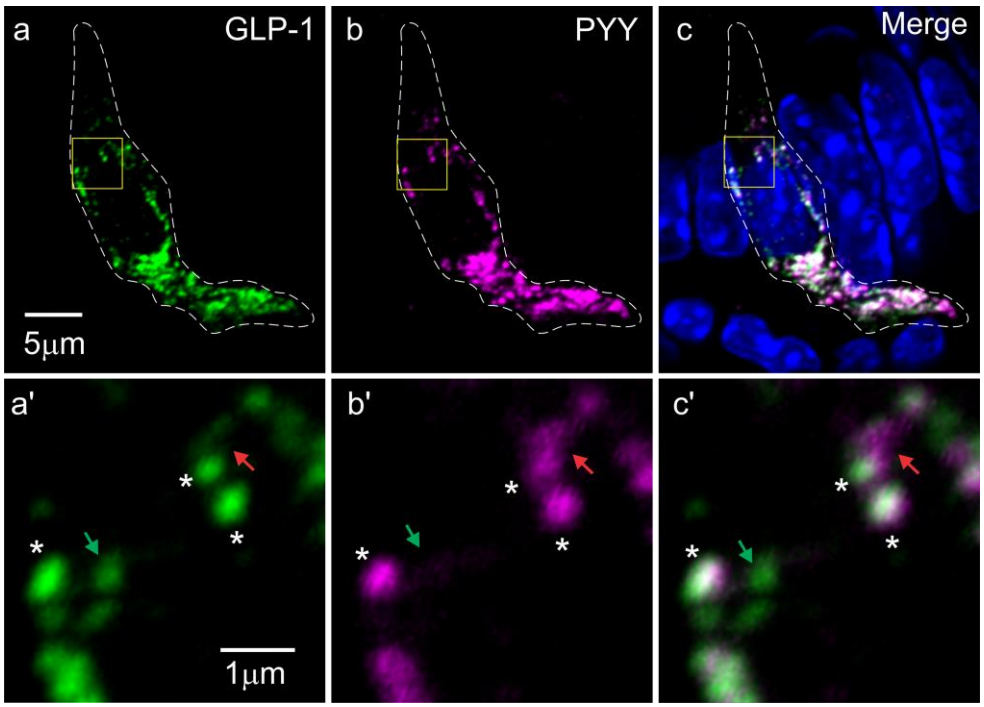
c'
d'


a
GLP-1
b
PYY
c
Merge
5μm
a'
b'
c'
1μm