**Title: Regulated Peristalsis into the acidic region of the *Drosophila* larval midgut is controlled by a novel component of the Autonomic Nervous System**

**Abbreviated title:** *Drosophila* peristalsis in larval midgut

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## ABSTRACT

The underlying cellular and molecular mechanisms that regulate and coordinate critical physiological processes such as peristalsis are complex, often cryptic, and involve the integration of multiple tissues and organ systems within the organism. We have identified a completely novel component of the larval autonomic nervous system in the *Drosophila* larval midgut that is essential for the peristaltic movement of food from the anterior midgut into the acidic region of the midgut. We have named this region the Superior Cupric Autonomic Nervous System or SCANS. Located at the junction of the anterior and the acidic portions of the midgut, the SCANS is characterized by a cluster of a novel neuro-enteroendocrine cells that we call Lettuce Head Cells, a valve, and two anterior muscular tethers to the dorsal gastric caeca. Using cell ablation and ectopic activation via expression of the *Chlamydomonas reinhardtii* blue-light activated channelrhodopsin, we demonstrate that the SCANS and in particular the Lettuce Head Cells are both necessary and sufficient for peristalsis and perhaps serve a larger role by coordinating digestion throughout the anterior midgut with development and growth.

**INTRODUCTION**

All higher metazoans have evolved digestive systems that extract sustenance from the environment for growth and survival.  Although digestive systems are adapted to diverse feeding behaviors, they share an overall similarity in their organization and all coordinate nervous system and endocrine input to govern the movement and the processing of food within the alimentary canal[1,2,3]. In vertebrates, the hypothalamus controls energy homeostasis, feeding, and organism-wide metabolic control[4].  In response to an empty stomach, the 'hunger' hormone ghrelin stimulates AMP-kinase activity in the hypothalamus resulting in an increase in feeding activity as well as increasing the motility of the intestine; the gut hormone leptin has the opposite effect on AMP-kinase activity in the hypothalamus and signals satiation[1,2,3,4]. The *Drosophila* larval gut contains all of the features of a complete alimentary canal[5,6], however the molecular and cellular mechanisms that regulate the digestion within the *Drosophila* larval midgut remains cryptic. Although many fundamental elements have yet to be discovered in *Drosophila*, a complex picture involving the roles of nutrient acquisition and key developmental events is emerging[7,8].  Homologues to several peptide hormones have been identified and many have clear roles in integrating growth and behavior with metabolism and nutrient acquisition[9-13].

To identify new cell types that function in digestion, we examined 37 Gal4 enhancer traps reported to express in the *Drosophila* larval midgut.  Nineteen of these Gal4 enhancer traps expressed in various patterns throughout the larval midgut (Supplemental Table 1; Supplemental Figure 1). In fifteen of these enhancer traps we noticed a distinctive cluster of cells at the junction between the anterior midgut and the

acid secreting portion of the larval midgut (Figure 1A). In four enhancer traps,

*ChaGal4, DdcGal4, DJ752Gal4, and MJ12 Gal4*, the expression was found exclusively

in these cells in the midgut proper (Supplemental Table 1).  The cells were bottle-

shaped and projected an apical, lamellipodial head into the lumen of the gut, thus we

named them Lettuce Head Cells (LHC; Figure 1B).  We observed an average of 7±2

LHC (n=37 midgut) per midgut. Each cell extends through the endothelial epithelium

and is associated with the overlying longitudinal muscle (Figure 1C). The LHC are

located at a conspicuous and persistent U-bend in the midgut in the area where the

allatostatin B/MIP endocrine cells can be found[14]. The LHC express several neural

markers including Choline acetyltransferase (Cha) and Dopa decarboxylase[15,16].

Although the anterior proventriculus and surrounding anterior midgut are innervated by

the ventral ganglion[17], no ventricular ganglion axons project into the SCANS. Thus the

LHC remain distinct from the rest of the nervous system (LaJeunesse, personal

observation).  We believe that the LHC are a new neuro-enteroendocrine cell type that

defines a new component of the larval autonomic nervous system that we have named

the Superior Cupric Autonomic Nervous System, or SCANS region. In addition to the

LHC, the SCANS contains a valve, which manifests as a thickening of the endothelial

lining (Figure 2D), and a pair of muscular tethers (Figure 2A and 2B).

To determine the role of the LHC in SCANS function, we ablated the LHC from

the larval midgut using a system based on the ectopic expression of either the

proapoptotic gene *UAS reaper* [11] or the castor bean toxin *UAS ricin*[19,20]. We directed

the expression of these ablation genes using the *Gal80*[ts] conditional expression

system[21,22] and two Gal4 Drivers specific to the LHC, *ChaGal4* and *DJ752*. Larval

midguts with ablated LHC were assessed in four separate assays: (1) a feeding assay to examine the movement of food through the alimentary canal; (2) a feeding assay in which the composition of the acidic portion of the midgut was assessed; (3) a morphological assay in which we examined the structure of the midgut; and (4) a functional assay in which we observed peristalsis within the SCANS region.  Although ablation of the LHC did not change the passage of food through the gut (see Supplemental Table 2) or generate any morphological changes to the SCANS (see Supplemental Table 3), we observed significant changes in peristalsis in the SCANS region and in the composition of the acidic region of the larval midgut.

In LHC ablated midguts, we observed a significant decrease in the number of contractions versus the controls (Table 1; compare Supplementary Video 1 with Video 2). Loss of the LHC resulted in greatly reduced peristalsis when compared to controls (Table 1). In addition to LHC-ablated SCANS, we observed a significant reduction of peristalsis after knocking-down *Cha* expression using a *UASRNAiCha* construct (Table 1), thus demonstrating a definitive requirement for *Cha* in the LHC.

To further examine the role of the LHC in peristalsis we ectopically activated LHC by expressing the *Chlamydomonas reinhardtii* Channelrhodopsin-2 (ChR2) in the LHC and examining the effect on peristalsis.  The ChR2 protein is a light-activated cation-selective ion channel that, when expressed in a muscle or neuron and exposed to blue light ($\lambda$~488nm), will initiate an action potential[23].  We showed that the LHC are not only necessary but also sufficient for the generating the peristaltic wave within the SCANS region. In the SCANS we observed a significant increase in the number of contractions in midguts with LHC expressing the CHR2 protein when exposed to blue

light versus white light (+43% for DJ752 Gal4 and +26% for ChaGal4; Table 2). These experiments along with the expression and requirements for *Cha* clearly demonstrate that the LHC are an excitable cell-type which directly activate visceral muscle peristalsis.

Although we found no change to the overall movement of food despite the ablation of the LHC, we examined the effects of loss of peristalsis digestion by evaluating the characteristic changes in pH within the larval midgut[24]. Typically, larvae fed food containing 2% Bromophenol Blue will have an anterior midgut with a deep blue food and an acidic region that is marked by bright yellow food. We observed a significant alteration in the pattern of pH in the acidic compartment of midguts missing the LHC Ablation or in midguts with knocked-down *Cha*. This result was manifest as an increase in the number of larvae with green instead of yellow food in the acidic region (Table 3). Our results show that LHC-mediated peristalsis at the SCANS region is required for maintaining the proper pH within the acidic region, perhaps by regulating the entry of food into the acidic compartment of the larval midgut. The significance of this change in food pH is unclear; however larvae with ablated LHC have a slight delay (1-2 day) in pupation (data not shown) suggesting that a reduced efficiency of nutrient procurement by the gut.

The LHC are neural-type of enteroendocrine cells, and in both human and insect systems enteroendocrine cells have been shown to secrete a number of hormones that govern digestion and growth [1,14,24]. For instance, in the human duodenum enteroendocrine cells called L-Cells regulate the movement of food through the gut via the secretion of glucagon-like peptide-1 in response to the presence of glucose[26]. The

highly ruffled lamellipodial endings of LHC (Figure 1B) suggest that they too may be sensory, perhaps responding to specific products of digestion to initiate peristalsis directly through the direct stimulation of visceral muscle peristalsis in the SCANS region.  This signal may be some digestive cue in the anterior midgut that triggers entry into the acidic region of the midgut, or perhaps the signal is a reflux/retrograde signal from the acidic region that keeps the valve closed.

The most curious portion of the SCANS region is the muscular links from the SCANS to the gastric cecae. The gastric cecae emerge from the anterior midgut just posterior to the proventriculus as four blind-ended tubes[18].  The tips of the dorsal gastric cecae are linked to the SCANS region via modified longitudinal muscles (Figure 2A). The gastric caeca/SCANS muscular tether is extremely labile and typically destroyed during dissection, although the remnants are always observed with both the gastric caeca and SCANS (Figure 2B). These muscular tethers are extensions of the inherent longitudinal muscles of the dorsal gastric caeca (see Figure 2E). These tethers retain a striated organization of actin and myosin at the attachment to the midgut and at the origin on the gastric caeca (data not shown); however, the striated organization becomes less organized in middle of these muscles.  These muscle tethers express higher levels of Disc Large which can be found in internal plaques along the length of the muscle (Figure 2C). In a previous study these muscles have been suggested to be structural suspensor muscles[14].  However, given their connection to the SCANS region we believe that they may play a far more interesting role and could potentially relay signals between the SCANS and the proventriculus/anterior midgut.  The proventriculus functions as a valve for food input

into the midgut from the foregut[6,17] and therefore may have to be directly regulated by digestive signals from deeper within the gut. Although the function of the gastric cecae remains unknown, it has been suggested that the gastric cecae secrete digestive enzymes[18]; therefore, the connection of the SCANS with the gastric caeca could provide a physical means by which the secretion of putative digestive enzymes are adjusted during digestion from signals deeper within the gut.

The proventriculus is innervated by the ventricular ganglion[17] and thus signals from the CNS may be passed down into the SCANS region via these muscular tethers to regulate its valve/peristaltic function. The signal may be either mechanical, via a muscle contraction, or electrical, via an action potential. There is a precedent for the latter example; in vertebrate hearts, non-contractile muscular Purkinje fibers disperse electrical stimuli from the conduction system to the ventricular cardiac muscles[27]. Given the ontological and structural similarities between the vertebrate cardiac system and the *Drosophila* larvae visceral muscle[28,29], this remains an intriguing possibility. Regardless, the linkage between the SCANS and the dorsal gastric caeca suggests that the entire anterior midgut, from the proventriculus to the SCANS region, may operate as a single unit during early digestion. The SCANS region is a novel structure which plays a key regulatory role in the assessment of digestion in the anterior midgut and perhaps also in mechanisms that coordinate digestion with development and growth in *Drosophila* larvae.

## METHODS

*__Drosophila__ Genetic Strains:* The stocks used in this study are as follows:  $w^{1118}$ (as wild-type), *DJ752, ChaGal4, UAS::CD8GFP, UAS::ricin* (courtesy of K.G. Moffat, University of Warwick), *UAS::rpr.c* and *Ubi::Gal80$^{ts}$; UAS::ChR2 X2* (courtesy of Andre Fiala, Department of Genetics and Neurobiology, Theodor-Boveri-Institut, Julius-Maximilians-Universität Würzburg).

**Immunohistochemistry:** 5 day old larvae were dissected in 1X PBS containing freshly made 4% paraformaldehyde; larvae were fixed for 3 hours, washed, and mounted.  We used the anti-Disc Large antibody, monoclonal 4F3 (Developmental Studies Hybridoma Bank; 1:1000). For actin visualization we used Alex 564 phalloidin (Molecular Probes; 1:2000). All micrographs were imaged using an Olympus IX81 inverted FV500 confocal microscope.

**LHC ablation, ChR2 LHC Gain of Function experiments and movies:** LHC ablation was determined by the early (1 hour) GFP expression and latter (4-6hour) loss of expression of a *UAS CD8GFP* reporter gene. To image the peristalsis in the SCANS region, larval midguts were dissected in S2 cell media and mounted on a glass slide with a coverslip with clay feet to prevent compression. A series of 120 images (2 images per second) were captured with a UPlanFI 20X dry objective using a CoolSnap CCD camera mounted on an Olympus BX51 upright compound microscope. Data was compiled with ImagePro software.  Statistical analysis was performed using a two tailed T-Test assuming equal variances in Microsoft Excel. For the gain of function experiments, larvae of the appropriate genotype were cultured overnight on food containing 100mM all-trans retinal (Sigma, R2500) and imaged the next day as above

with one exception: the larvae were imaged under white light for thirty seconds, and then under an oscillating blue light (488nm; 2Hz) for another thirty seconds.

**Green Food/Yellow Food assay:** 5 day old larvae were cultured overnight in food containing 2% Bromophenol Blue, which is blue in basic solution and yellow in solutions with a pH lower than 3.0. Larvae were then dissected and the color of the acidic portion of their midgut noted.

**Author Contributions**: DRL wrote the manuscript, supervised the research presented, assisted in the execution of all experiments and experimental designs described in this paper. BJ performed in the LHC ablation experiments and the ectopic activation experiments. JP and KK collected confocal images of the Gal4 enhancer traps and images of the SCANS region and assisted in writing of the manuscript, GZ assisted in the generation of movies for the ablation and ectopic activation experiments as well as the green/yellow food experiments.

**Figure legends:**

Figure 1: The SCANS region and Lettuce Head Cells.  A) The SCANS region as shown by *ChaGal4* expression. Arrows point to individual Lettuce Head Cells (LHC). Left is towards the anterior/proventricular portion of the midgut, towards the right of these cells is the acidic region. The lumen is defined by autofluorescence of the yeast within the food. B). A confocal micrograph up of an individual LHC. The cell is oriented apical on the left and basal to the right. The apical head with its lamellipodial ruffles (arrow) extends into the lumen and sits on top of a disc of filamentous actin. C). Within the SCANS, babGal4 expresses in the LHC (thick arrows) and in the longitudinal muscles (thin arrows). D) The LHC (large arrow), although associated with the longitudinal muscles, is not in contact with the longitudinal visceral muscles (small arrow heads). Note the space between these two cells.

Figure 2: Organization of the SCANS region. A) Staining with anti-Discs large antibody shows an extension of the longitudinal muscle (large arrow) from the dorsal Gastric cecae (gc) inserts into the SCANS region adjacent to the LHC (green *UAS CD8GFP/DJ752 Gal4,* thin arrows). Another (broken) attachment is visible just below this one (medium arrow). The muscular tethers expresses higher levels of the functional protein Discs Large. B) Staining with anti-Discs large shows a remnant of the tether (large arrow) inserting into the musculature of the SCANS. In green (*UAS CD8GFP/DJ752 Gal4,* thin arrows), an LHC inserts through the endothelial wall into the lumen of the gut.  The red cells (arrow heads) are yeast within the lumen of the intestine. C) Within the modified longitudinal musculature tether, intracellular plaques of Disc Large Protein are arranged lengthwise (arrows). D) In red (actin via Alexa546 phalloidin), a thickened endothelial wall results in a "valve" (large arrow heads), just distal to the LHC (thin arrows, in GFP). Off to one side, the muscular tether (small arrow head) is visible. E) Schematic of the SCANS region.  Two dorsal gastric cecae (dgc) and two ventral (vgc) project from the midgut just below the proventriculus (pro). Along the two dorsal gastric cecae snakes a longitudinal muscle (in red) which projects from the tip and attaches to the SCANS region. Inset: the SCANS region in detail, the muscular tether from each gastric cecae, the LHC (in green) and the thickened endothelial valve (in red).

**Table 1:** Ablation of the LHC from SCANS alters peristalsis

| Genotype | Condition/Temperature treatment | n | Average # of contractions per minute ± SD |
|---|---|---|---|
| *w^1118* | **29°C o/n; RT 3 hours** | 33 | 10.7 ± 3.1 |
| *UAS ricin/+; DJ752Gal4/Gal80^ts* | **29°C o/n; RT 3 hours** | 30 | 2.6 ± 2.8*^A |
| *UAS reaper/+; DJ752Gal4/Gal80^ts* | **29°C o/n; RT 3 hours** | 20 | 2.0 ± 2.7**^A |
| *UAS ricin/+; Gal80^ts/+ (control)* | **29°C o/n; RT 3 hours** | 20 | 10.2 ± 2.9 |
| *UAS reaper/+; Gal80^ts/+ (control)* | **29°C o/n; RT 3 hours** | 15 | 10.5 ± 2.1 |
| *DJ752Gal4/+ (control)* | **29°C o/n; RT 3 hours** | 15 | 9.6 ± 4.1 |
| *w^1118 (wild type)* | **RT** | 43 | 10.1 ± 3.9 |
| *UAS ricin/+; DJ752Gal4/Gal80^ts* | **RT** | 20 | 14.5 ± 5.9 |
| *UAS reaper/+; DJ752Gal4/Gal80^ts* | **RT** | 20 | 8.54 ± 4.5 |
| *DJ752Gal4; UAS ChaRNAi* | **RT** | 16 | 1.3 ± 1.58***^A |
| *UAS ChaRNAi* | **RT** | 10 | 10.1 ± 2.1 |
| *UAS Ddc RNAi* | **RT** | 8 | 9.7 ± 1.5 |

* significance P=2.5E-16 when compare to w1118 (wild type) 29°C o/n; RT 3 hours control
** significance P=4.56E-14 when compare to w1118 (wild type) 29°C o/n; RT 3 hours control
***significance P=2.89E-12 when compare to w1118 (wild type) RT control
^A - "contractions" in these classes were uncoordinated twitches within the visceral musculature.

**Table 2.** Activation of the LHC is sufficient to induce ectopic contractions in the SCANS region.

| Genotype | n | White light contractions/min | Blue light contractions/min | % change |
|---|---|---|---|---|
| *w^1118* | 20 | 12.7 ± 5.5 | 12.8 ±2.7 | +0.8% |
| *DJ752 Gal4; UASChR2X2(with retinal)* | 20 | 11.5 ± 4.2 | 16.4 ± 4.0 | +43% * |
| *Cha Gal; UASChR2X2 (with retinal)* | 20 | 9.7 ± 4.5 | 12.2 ± 4.0 | +26% ** |
| *DJ752 Gal4; UASChR2X2 (no retinal)* | 12 | 11.3 ± 3.6 | 9.2 ± 3.5 | -19% |
| *Cha Gal4; UASChR2X2 (no retinal)* | 14 | 11.1± 4.0 | 10.3± 5.0 | -7% |
| *DJ752 Gal4 (control)* | 16 | 9.1 ± 5.0 | 9.1± 4.5 | 0% |
| *Cha Gal4 (control)* | 12 | 9.7±4.3 | 8.3±3.4 | -14% |
| *UASChR2X2 (control)* | 9 | 11.5±3.6 | 11.5±3.6 | 0% |

* Significant increase in number of contractions during exposure to Blue Light when compared to the number of contractions during exposure to white light, P=1.7E-4
**Significant increase in number of contractions during exposure to Blue Light when compared to the number of contractions during exposure to white light, P=6.4E-4

**Table 3. Loss of LHC results in Green food in the acidic region**

| Genotype | Condition/Temperature treatment | n | % yellow food in acidic region | % green food in acidic region |
|---|---|---|---|---|
| $w^{1118}$ | 29°C o/n; RT 3 hrs | 16 | 94% | 6% |
| UAS ricin/+; DJ752Gal4/Gal80$^{ts}$ | 29°C o/n; RT 3 hrs | 30 | 27% | 73% |
| UAS reaper/+; DJ752Gal4/Gal80$^{ts}$ | 29°C o/n; RT 3 hrs | 21 | 38% | 62% |
| UAS ricin/+; Gal80$^{ts}$/+ (control) | 29°C o/n; RT 3 hrs | 11 | 91% | 9% |
| UAS reaper/+; Gal80$^{ts}$/+ (control) | 29°C o/n; RT 3 hrs | 11 | 82% | 18% |
| DJ752Gal4/+ (control) | 29°C o/n; RT 3 hrs | 30 | 97% | 3% |
| UAS ricin/+; DJ752Gal4/Gal80$^{ts}$ | RT | 17 | 100% | 0% |
| UAS reaper/+; DJ752Gal4/Gal80$^{ts}$ | RT | 14 | 100% | 0% |
| DJ752Gal4/+ | RT | 12 | 100% | 0% |
| DJ752Gal4; UAS ChaRNAi | RT | 19 | 53% | 47% |
| UAS ChaRNAi | RT | 12 | 92% | 8% |

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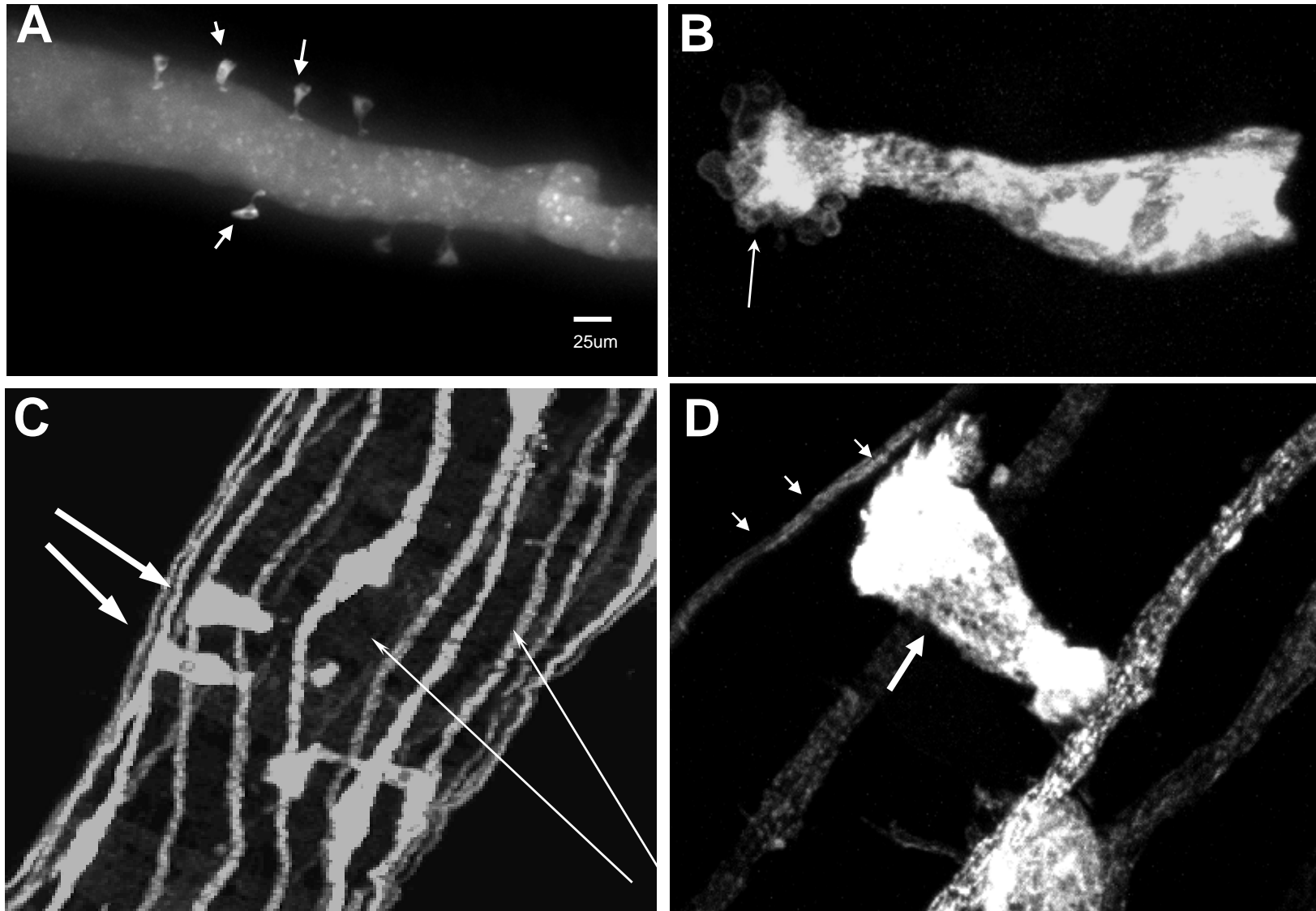| 16 | Konrad, K.D., Marsh, J.L. (1987). Developmental expression and spatial distribution of dopa decarboxylase in Drosophila. Dev. Biol. 122(1)**:** 172--185. |
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# Figure 1: The SCANS region and Lettuce Head Cells.

# Figure 2: Organization of the SCANS region