Drosophila Peptide Hormones Allatostatin A and Diuretic Hormone 31 Exhibiting Complementary Gradient Distribution in Posterior Midgut Antagonistically Regulate Midgut Senescence and Adult Lifespan

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INTRODUCTION

In a wide variety of metazoan animals, the intestine is composed of various types of cells in addition to enterocytes (ECs), which are responsible for nutrient absorption, a common function of the intestine. Enteroendocrine cells (EEs) are known to be a type of evolutionarily conserved intestinal cells and show a scattered distribution among many ECs within the intestinal epithelium. The EEs secrete a number of hormones to the basal side of the intestinal epithelium in response to various environmental stimuli including nutrients and bacteria in the intestinal lumen. Both in mammals and insects, EEs are classified into several subtypes based on the hormones they produce. However, some hormones are produced in multiple EE subtypes while others are produced in a single EE subtype. We do not sufficiently understand why these differences in production site are required for hormone synthesis.

Drosophila melanogaster is an excellent model organism that is used in various studies in developmental biology to study gene functions. The Drosophila adult midgut is similar to the mammalian small intestine with a single layer of epithelial cells, except that it does not have a crypt-villi architecture. In fact, epithelial cells are composed of intestinal stem cells (ISCs), enteroblasts (EBs), ECs, and EEs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), the cellular lineage of which has been extensively investigated and is similar among phylogenetically distant organisms (Fig. 1A). Additionally, the Drosophila intestinal epithelium is surrounded by visceral muscles (VMs), nerves, and the trachea (Fig. 1B) (Jiang et al., 2009; Zeng et al., 2010; Buchon et al., 2013), which also create a surrounding environment and...
The *Drosophila* midgut EEs are known to express six or more types of peptide hormones (Veenstra et al., 2008; Veenstra, 2009a, b; Veenstra and Ida, 2014). It has also been reported that, in a region known as the adult posterior midgut (PMG, corresponding to the mid-R4 to R5 region by Buchon et al., 2013), EEs usually occur in pairs that express two different peptide hormones in each cell (Ohlstein and Spradling, 2006). One hormone is Allatostatin A (AstA) and the other is Tachykinin (Tk). However, in later studies, it was elucidated that Tk-producing EE subtypes simultaneously express Diuretic hormone 31 (Dh31, Fig. 1B) (Beehler-Evans and Micchelli, 2015). Accordingly, PMG EEs are classified into two subtypes: AstA and Tk/Dh31. All these hormones are also known to be expressed in brain neurons and/or neurosecretory cells (NSCs). However, unlike in the previous report (Ohlstein and Spradling, 2006), we noted that these two types of PMG EEs show discriminative distribution along the antero-posterior axis (this study, Fig. 1C, Supplementary Figures S1, S2 online). The function of the midgut-producing fraction of these hormones has not been studied, and the significance of differential expression between these two hormones in each EE subtype is not known.

Octa- to tridecapeptide Allatostatin were first isolated from the brain of a cockroach, *Diploptera punctata*, based on their ability to repress the juvenile hormone-synthesizing activities in the copora allata, and their primary structures were elucidated (Woodhead et al., 1989). However, their *Drosophila* homologs do not show this function, but instead regulate various physiological activities, such as feeding, peristalsis, cation transport, and diuretic action (Veenstra, 2009b; Lajeunesse et al., 2010; Hergarden et al., 2012; Vanderveken and O’Donnell, 2014; Hentze et al., 2015). Dh31, a peptide hormone with 31 amino acid residues, was also

Fig. 1. AstA and Dh31 expression and their specific knockdowns preferentially in adult PMG. (A) Cellular lineage of various midgut cell types derived from ISC in *Drosophila* adult. Orange circles in the cells represent nuclei. (B) Schematic diagram of cell location in longitudinal section of adult midgut epithelium. Orange ellipses and gray bar beneath the cells represent circular muscles and basement membrane, respectively. (C) Location of PMG in midgut and complementary distribution of AstA and Dh31 subtypes of EE. According to the nomenclature of midgut subregions by (Buchon et al., 2013), the PMG we focused on corresponds to the subregion from the central position of R4 to the posterior end of R5. The entire midgut is depicted according to Fig. 1 in (Buchon et al., 2013). The photograph shows a close-up view of the PMG where posterior regions are enriched with AstA and Dh31 cells, respectively (an enlarged photograph is shown in S Fig. 2). (D–I) High-power images of PMG from 7-day-old adult. Expression of pros\(^{\text{GAL4}}\) (Green in (D–I)), monitored with UAS-GFP, was detected in all EEs of the central region of the PMG. Subtypes of EEs were marked with anti-AstA antibody (magenta) and anti-Dh31 antibody (cyan). (E, G, and I) Close-up views of boxed areas in (D, F, and H), respectively. pros-positive EEs frequently occurred in pairs. (D–E’) One cell in pair of EEs expressed AstA (magenta) and the other expressed Dh31 (cyan). (F–G’) When AstA was knocked down (pros > AstA\(^{\text{IR}}\)), AstA staining was specifically eliminated and Dh31 staining remained unchanged. (H–I’) When Dh31 was knocked down (pros > Dh31\(^{\text{IR}}\)), Dh31 staining was specifically eliminated and AstA staining remained unchanged. Scale bars: 50 μm in (D) and also (F) and (H); 100 μm in (B).
found in *Dipterota punctata* based on its diuretic activity (Furuya et al., 2000); its vertebrate homolog calcitonin was previously known. The functions of the *Drosophila* homolog are known to regulate diuretic activity, peristalsis, cation transport, and sleeping (LaJeunesse et al., 2010; Kunst et al., 2014; Vanderveken and O’Donnell, 2014). It has been suggested that AstA and Dh31 show partial antagonism with regard to their various functions mentioned above (Veenstra et al., 2008; Veenstra, 2009b; Vanderveken and O’Donnell, 2014). In the present study, we investigated such antagonism in the regulation of longevity and senescence, and found that the adult lifespan is shortened by AstA inhibition, but extended by Dh31 inhibition through midgut EE-preferential RNA interference (RNA). By AstA knockdown, premature senescence of the young midgut was also observed at the same time. Contrary to, by Dh31 knockdown, delayed aging in old midguts was obvious. Through detailed analyses, we describe and discuss the antagonistic roles of these two hormones, which may account for the differential-expression characteristics of the AstA- and Dh31-subtypes of EEs.

**MATERIALS AND METHODS**

**Upd3-RedStinger reporter**

To generate an *unpaird3* (*upd3*)-RedStinger reporter, a genic fragment containing the *upd3* promoter region (Jiang et al., 2011) was amplified with polymerase chain reaction (PCR) and digested with *Bgl II/Xho I* to insert between the same restriction sites of the *pRed-H-stinger* vector. Transgenic lines were established through standard P-element-mediated transformation.

**Fly strains**

The following fly stocks were obtained from Kyoto Stock Center (DGRP) of Kyoto Institute of Technology: *w^1^* (wild-type), *NP6267* (*esg-GAL4*) (Hayashi et al., 2002), *NP1* (*MyoI-ALAG4*) (Jiang et al., 2009), *Ay-GAL4* (also known as Act5c>y>Gal4), *UAS-GFP* (Furuya et al., 1997), *rp49-GAL4* (Jiang et al., 2009), and *rp49-FLP* (Bloomington Stock Center: #8163) (Britton et al., 2002); and Vienna *Drosophila* RNAi Center: *UAS-AstA^IR^* (v103215), *UAS-Dh31^IR^* (v37764 and v50296), and *UAS-AstA-R^IR^* (v101395). The hs-FLP provides flipper recombinase (FLP) under heat-shocked condition. The *tub-gal80^c^* lines ubiquitously express a temperature-sensitive GAL80 protein, which represses GAL4 at the restrictive temperature (19°C), but does not repress it at the permissive temperature (29°C). The *prospero^V1^* (*prospero^V1^*)-GAL4 (*pros-GAL4*) reporter was a generous gift from Dr. Jean-Francois Ferveur (Balakireva et al., 1998), *prospero^{D^{3}-L^{3}}* (*pros-lacZ*) was a generous gift from Dr. Volker Hartenstein, *Delta-GAL4* (*Dil-GAL4*) and *Sui/H^{G}B^{E}-GAL4* lines were generous gifts from Dr. Steven Hou (Zeng et al., 2010), and *midgut expression 1* (*mes^1)^Y^*)-GAL4 lines were generous gifts from Dr. Graham Thomas. The *UAS-GATA^E^* was described in (Okumura et al., 2016). All the fly genotypes used in this study are listed in Supplementary Table S1.

**FLP-out system**

The FLP-out system (Ito et al., 1997) in the adult midgut to create mosaic areas where ectopic gene expression was artificially induced. The adult flies with the desired genotype were reared at 25°C for 2 days after eclosion. The flies were then heat-shocked twice at 37°C for 20 minutes in a heat-water bath and cultured at 25°C for appropriate periods (seven days). The mosaic areas with ectopic gene expression were marked with UAS-GFP expression by the connected Act5c-GAL4 driver created by FLP-mediated cutting out (FLP-out) of the intervening sequence between two FLP recombinase targets (FRTs).

**Immunostaining**

A dissected female adult midgut at an appropriate adult stage was fixed with 4% paraformaldehyde at room temperature for 40 min. After washing with phosphate-buffered saline containing 0.1% TritonX-100, immunostaining was carried out with the following primary antibodies: rat anti-GFP (Nacalai Tesque, 1:200 dilution), rabbit anti-RFP (Clontech, 1:200 dilution), chicken anti-beta-galactosidase (abcam, 1:200 dilution), rabbit anti-phospho-histone H3 (pH3) (Upstate Biotech, 1:200 dilution), mouse anti-Prospero (DSHB, 1:100 dilution), mouse anti-AstA (DSHB,1:10) (Woodhead et al., 1989), mouse anti-Dl (DSHB, 1:100 dilution), and rabbit anti-Dh31 (1:500 dilution, a generous gift from Dr. Jan Veenstra) (Veenstra et al., 2008). The secondary antibodies used were Alexa Fluor647 or Alexa Fluor555-conjugated anti-mouse IgG (Jackson Immuno Research, 1:200 dilution), Alexa488-conjugated anti-rat IgG (Jackson Immuno Research, 1:200 dilution), Alexa Fluor555 or Alexa Fluor647-conjugated anti-rabbit IgG (Jackson Immuno Research, 1:200), and DyLight649-conjugated anti-chicken IgY (Jackson Immuno Research, 1:200 dilution). Nuclei were stained with 4’6-diamidino-2-phenylindole (DAPI, SIGMA).

**Imaging using laser confocal microscope**

For all the immunofluorescence images, the Digital Eclipse C1Si laser confocal system (Nikon) was used. For stacked images of the multiple confocal Z-sections of the adult brains (Supplementary Figure S3), 44–45 sections covering whole brains with 1-μm-depth intervals were obtained by very weak intensities to avoid saturation of fluorescence for quantitative observation. These fluorescent images were similarly obtained among three genotypes (*pros-GAL4, pros > AstA^IR^*, and *pros > Dh31^IR^*).

**Discrimination of diploid and polyploid DI-positive cells in adult midgut**

The adult midguts in each genotype at appropriate stages were stained for Dl and DAPI, as described above. The midgut from normal young adults shows DI expression only in the diploid ISCs that belong to the smallest cell population in the midgut accompanied with EBs and EEs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Under this condition, the large cells are the developing and mature ECs, the nuclei of which are at tetraploid, octoploid, and larger polyploid levels (Zielke et al., 2014) easily distinguishable from the diploid cell population. If the cells with polyploid nuclei express DI, we judged them as abnormal DI expression by senescence.

**Transmission electron microscopy (TEM)**

An adult midgut was dissected out and fixed with 2% glutaraldehyde/0.1M phosphate buffer and subsequently with 2% OsO4/0.1M phosphate buffer. After fixation, the tissue was embedded in Quetol-812 resin (Nisshin-EM) and sliced into ultrathin sections of 70-nm thickness. The ultrathin sections were stained with uranyl acetate-lead citrate. The transmission electron micrographs were obtained using JEM-1200EX (JEOL) with 80-kV accelerating voltage.

**Analysis of AstA and Dh31 mRNAs in adult brains and guts by reverse transcription-polymerase chain reaction (RT-PCR)**

Four adult female brains and guts of each genotype at seven days after eclosion were dissected on ice. Total RNA was isolated using the NucleoSpin RNA XS Kit (TaKaRa), and cDNA was transcribed using the PrimeScript RT-PCR Kit using oligo (dt) primer (TaKaRa). Expressions of genes were normalized using rp49 as a reference gene. The nucleotide sequences of the PCR primers were as follows: *rp49-Fwd, ATCGGGTACGGATCGAACAA; rp49-Rev, GACAATCTCCTTGCGCTTCT; AstA-Fwd, TGTCAAC-
GTGCCACAGG; AstA-Rev, CACTCTGATCGATCTCGTTG; Dh31-Fwd, TGCAGTCAGCAGCAGTAACG; Dh31-Rev, TGGCATGTTTC-GCCCTCCTG.

In the reference assay by rp49, PCRs using equal amounts of total cDNA preparations from w1118 (control), pros > AstAIR (experiment), or pros > Dh31IR (experiment) were carried out with 24, 26, 28, 30, and 32 cycles at 94°C for 10 sec., 55°C for 1 min., and 72°C for 1 min. After reactions, each aliquot was electrophoresed in agarose-gel, stained with ethidium bromide and measured for rp49 band intensities by Image J. Concentrations of total template cDNA among the samples were adjusted by dilution. The PCRs for AstA or Dh31 were carried out as above, and their five microlitr aliquots were electrophoresed, stained, and photographed.

Analysis of upd mRNAs in adult midguts by RT-PCR

Four adult midguts (from two males and two females) of each genotype at 21 days after eclosion were dissected on ice. The RNA and cDNA were prepared, as described in the preceding section. The nucleotide sequences of the PCR primers were in accordance with (Karpowicz et al., 2010) as follows: upd1-Fwd, TGCACTCAGCATCCCAATCAG; upd1-Rev, ATAGTCAGCTGTTGCGTTGCC; upd2-Fwd, TGCTACCGTGGAGGCTTCG; upd2-Rev, GACTCTTCTCCGGCAAATCAG; upd3-Fwd, AAATTGAATGCCAGCAGTACG; and upd3-Rev, CCTTCTGTTGCGTTGCCTTC.

A reference assay was carried out, as described in the preceding section. The PCRs for upd1, upd2, and upd3 were carried out and analyzed, as described in the preceding section.

Description of adult survival curve

Adult flies were reared at 25°C on standard yeast-glucose-cornmeal-agar medium that was changed every 2–3 days, and surviving flies were counted every five days according to the standard protocol (Clancy et al., 2001; Hwangbo et al., 2004).

Senescence analysis by senescence-associated β-galactosidase activities

Senescence-associated β-galactosidase activities derived from lysosomes were assayed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology Inc.) (Goldstein, 1990; Dimri et al., 1995).

RESULTS

Distribution of two subtypes of EEs in PMG

The EEs in the midgut show a tendency to distribute as pairs, where each EE is in a closed position. As previously described (Ohlstein and Spradling, 2006), some pairs are composed of single AstA- and Dh31-subtype cells (Fig. 1E, F). However, the region displaying this feature is restricted to the central part of the PMG. In fact, the Dh31- and AstA-subtype cells dominate in the anterior and posterior positions in the PMG, respectively (Fig. 1C, Supplementary Figures S1, S2). In these end regions, both EEs are frequently the same subtype. Consequently, there are two gradients with opposite slopes in the frequencies of each EE subtype (Fig. 1C, Supplementary Figure S1).

Midgut-preferential knockdown of AstA- or Dh31-subtype EEs

The major sources of both AstA and Dh31 are found both in the brain and midgut. In view of the immunocytochemical staining pattern of the brain and previous reports (Hergarden et al., 2012; Kunst et al., 2014; Hentze et al., 2015), the brain AstA and Dh31 are released as neurotransmitters from various neurons and as neurohormones from several large NSCs (Supplementary Figure 3A). Targeting of the AstA neurons to insulin-producing cells (IPCs) was also documented (Hentze et al., 2015). Unlike this connection, the intestinal fraction of AstA is always released from the EEs that have no projection targeted to other cells (Supplementary Figure 3D).

To examine the functions of the midgut-fraction of AstA and Dh31, we carried out midgut-preferential RNAi for each gene. We used prospero (pros)-GAL4 allele V1, which shows an expression in midgut EEs as well as in the central nervous system, while other pros-GAL4 alleles do not show expression in midgut EEs. We first planned to restrict the period of RNAi induction to the adult stage by using a temperature-sensitive version of GAL80, a GAL4 repressor (GAL80*, McGuire et al., 2003). However, these flies exhibited morphological and cytological phenotypes indistinguishable from those without restriction of RNAi period (data not shown). Therefore, in later experiments for phenotypic
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analyses, we did not use GAL80ts and induced RNAi throughout the lifespan. We found that the AstA-RNAi caused a specific decrease in AstA-positive cells in the PMG (Fig. 1F, G). We also carried out similar cell type-specific RNAi for Dh31 (Fig. 1H, I). With regard to the brain, the pros expression can be widely found in brain neurons but not in the large NSCs normally expressing AstA or Dh31 (Supplementary Figures S3A–A”). However, both in the neurons and NSCs in the flies where AstA or Dh31 was knocked down in the pros-expressing cells, we did not find marked decrease in both hormone levels (Supplementary Figure S3B–B”, C–C”). Also in the RT-PCR analyses, we did not detect marked decreases in the brain (Supplementary Figure S3D left) but detected decreases in the midgut (Supplementary Figure S3D right). These results indicate that the depletion of the midgut-hormonal fractions of these two peptides was preferentially achieved, although a local decrease in the brain neurotransmitter fraction that is undetectable through whole-brain RT-PCR analysis might also hold true.

Effect of hormone knockdown on adult lifespan and midgut senescence

The brain-producing fraction of various brain-gut peptides in Drosophila is known to affect various systemic functions such as feeding, sleeping, and peristalsis, as described above. To get a clue for the effect of the preferential inhibition of midgut hormones, we first measured adult lifespans as a systemic function. Consequently, the half-life period was 46 days in the control flies (green solid line in Fig. 2A, B), whereas it decreased to 34 days when AstA was knocked down (red solid line in Fig. 2A, B). When Dh31 was knocked down, however, the half-life period was extended to 63 days (blue solid line in Fig. 2A, B). The F1 progenies obtained by backcrosses of each genotype with the control fly strain (w1118) yielded results with similar traits (dotted lines in Fig. 2A, B).

However, when we used the GAL80ts to restrict the RNAi period to the adult stage, as described above, Dh31-knockdown flies showed a drastically short adult lifespan, which suggested that high temperature (29°C) raises other response(s) to shorten lifespan (data not shown). In fact, the expanded-midgut phenotype seen in the standard Dh31-knockdown flies (Supplementary Figure 7D) was further exaggerated (data not shown), which may severely interfere with urination and long lifespan. Because AstA and Dh31 have antagonistic functions in various aspects, high temperature should also be considered to affect AstA-knockdown flies.

From these results, we argue that the gut-producing fractions of AstA and Dh31 possess an antagonistic ability to affect adult lifespan, although involvement of the late-onset effect by knockdown before the adult stage has remained uncertain.

We then examined the progress in the senescence phenotype in each organ. We first compared the morphology of the midguts between each genotype on the 7th and 21st days after eclosion to examine the appearance of known senescence features: ISC-like tumor formation due to abnormal increase in the proliferation rate and ectopic expression of the ISC marker Delta (Di) in the polyploid early and mature

![Image of normally aged ISCs in pros-GAL4 (A), pros > AstAIR (B), and pros > Dh31IR (C) at 21st day after eclosion. Green represents EEs, magenta represents ISCs with anti-Dl antibody staining, and blue represents all nuclei with DAPI staining. In B”, three examples of the polyploid cells with ectopic Dl expression are indicated with yellow arrowheads. (D) Frequencies of mitotic (pH3-positive) ISCs in the PMG at 7th and 21st days after eclosion. Error bars are S.E.M., and values were calculated using Student’s t test. Asterisks indicate significant differences (**P < 0.01). Note that the number of pH3-positive cells in the Dh31-knockdown midgut was less than that in the control young midgut, suggesting an inhibitory effect of Dh31 knockdown on normal ISC proliferation (see text). (E) Image of normally aged ISCs in pros > GFP at 28th day after eclosion. High-frequency, hyper-proliferation, and polyploidization of ISCs were apparently found. Scale bars: 50 μm in (A) and also (B), (C), and (E).]
ECs (Biteau et al., 2008; Choi et al., 2008). Under normal conditions, all these phenotypes were found after around the 28th day (Fig. 3E). In the case of AstA knockdown, the above senescence phenotypes were prematurely observed on the 21st day (Fig. 3B), while in the case of Dh31 knockdown, ISCs were normally scattered on the 21st day, as observed in the control (Fig. 3A, C). These results indicate that the senescence levels of the midgut are affected by midgut hormones and correlate with individual longevity regulation.

In accordance with the tumor formation in AstA knockdown, frequencies of mitotic ISCs detected by the phospho-histone H3 (pH3)-antibody showed an abnormally earlier increase as a premature signature (Fig. 3D). In the Dh31-knockdown flies, an increase in the pH3-positive mitotic cells was not consistently observed until the 21st day, as observed in the wild type. Even in the young midgut of the Dh31-knockdown flies, the number of pH3-positive ISCs was less than that in the normal young midgut. This indicates that Dh31 has an inhibitory effect on ISC proliferation, although age-induced elevation of ISC proliferation has not been observed to date.

Site of AstA action in regulation of midgut senescence

We next examined the possibility of direct action of AstA on the midgut cells and, if so, which types of cells are targeted by these hormones by means of hormone-receptor AstA-R1 knockdown in a cell type-specific manner. The following GAL4 drivers were used; esg-GAL4 (ISCs/EBs), pros-GAL4 (EEs), NP1-GAL4 (ECs), 24B-GAL4 (VMs), DI-GAL4 (ISCs), Su(H)+GBE-GAL4 (EBs), and mex1-GAL4 (all midgut cells). When AstA-R1 was knocked down in ISCs/EBs or only EBs by either esg-GAL4 or Su(H)+GBE-GAL4, respectively, apparent ISC tumors arose and abnormally ectopic expression of DI was found in the polyploid cells (yellow arrowheads in Fig. 4C, F), which was similar to the phenotypes of the ligand AstA knockdown (Fig. 3B). However, knockdowns of AstA-R1 in other cell types in the midgut (Fig. 4A, B, D, E) were not affected. Quantitative comparison in senescence levels is shown in Fig. 4H. These results indicate that midgut senescence by AstA knockdown was caused by direct action to EBs.

Independence of adult lifespan and midgut senescence in action of AstA

Simultaneous occurrence of shortening lifespan and acceleration in midgut senescence led us to expect that midgut senescence reduces lifespan. We then measured the longevity of adults having the above senescence-accelerated midgut by AstA-R1 knockdown. Contrary to our expectation, these flies’ lifespans were not shortened (Fig. 4G), indicating that individual lifespan and midgut senescence are independent events. Furthermore, induction of this genetic mosaic after adult eclosion means that inhibition of AstA action only in the adult stage is enough to accelerate midgut senescence.
However, reduction in intestinal function by senescence progression should result in a decrease in nutrient absorption, which should affect longevity. To test for this, we generated model flies having a highly reduced function by knockdown of GATAe and measured their lifespan. GATAe is an evolutionally conserved family of transcription factors and known to regulate cell differentiation in various organs during development. GATAe is a fly homolog of mammalian GATA-4, -5, and -6, all of which belong to a GATA subgroup expressed in endodermal developing organs. GATAe is known to induce transcription of various genes showing gut-specific expression, such as beta-nu integrin, inxi7, mex1, and lambda trypsin (Okumura et al., 2007). Knockout or knockdown of GATAe led to interference in the differentiation of all the adult midgut cell types and a severe reduction in the whole gut structure (Okumura et al., 2016). Thus, GATAe is considered to be a master control gene of the midgut. The GATAe-knockdown flies showed a drastic decrease in adult longevity, probably due to insufficient nutrient intake (Supplementary Figure S4). This means that the flies in which AstA or AstA-R1 is knocked down have a premature senescent midgut, but its function in nutrient absorption is not greatly impaired.

Site of Dh31 action in regulation of midgut senescence and its epistatic to AstA action

Next, we focused on Dh31-Receptor (Dh31-R). In the 28th-day wild-type midgut described above, we observed normally aged phenotypes (Fig. 3E). When Dh31-R was knocked down in the EEs or VM, we found similar senescence phenotypes of the midgut to those in the wild type (Fig. 5B, C), which means that Dh31 does not act on these cell types, at least in the regulation of midgut senescence. By contrast, when Dh31-R was knocked down in the ISCs/EBs and ECs, only the ISCs, or only the EBs, delayed senescence phenotypes were observed (Fig. 5D–G), which were similar to the phenotypes of the ligand Dh31 knockdown on the 28th day (Fig. 5A). A quantitative comparison in senescence levels is shown in Fig. 5H. These results indicate that the delayed senescence of the midgut by Dh31 knockdown was caused by direct action on ISCs/EBs and ECs. These findings also raise a hypothesis in which the effects of AstA and Dh31 on midgut senescence are not mediated by insulin, a well-studied factor affecting lifespan and senescence. Moreover, simultaneous double knockdown of both AstA and Dh31 in the EEs showed a delayed senescence phenotype indistinguishable from that in the Dh31 singular knockdown midgut (Supplementary Figure S6). This means that Dh31 is epistatic to AstA and is consistent with the sites of action of these two hormones: the EBs (prospective ECs) as the sole AstA target are upstream of the ECs targeted by Dh31.

Difference in senescent responses between organs and independence of insulin signaling

We next verified whether other organs, such as the male accessory glands and Malpighian tubules, showed similarly aged phenotypes based on the observation of senescence-associated β-galactosidase activities (Goldstein, 1990; Dimri et al., 1995) in the lysosomes in 7th- and 28th-day adults. In the case of the male accessory gland, AstA knockdown unexpectedly did not accelerate senescence (Fig. 6C, D), although Dh31 knockdown lead to earlier senescence (Fig. 6E, F) in contrast to the case of the midgut. With regard to the Malpighian tubules, both AstK and Dh31 knockdowns did not show significant change in timing for the appearance of senescence phenotypes (Fig. 6I–L). From these observations, we concluded that the effect of AstA/Dh31 knockdown on the progression of senescence unpredictably varies between organs.

Insulin signaling is one of the main factors for lifespan control. In Drosophila, chico, a mutant of the insulin receptor substrate Chico, extends lifespan up to 141% (Clancy et al., 2001). As described above, however, the difference in the AstA/Dh31 effect on senescence progression between organs suggested that hormones circulating in the hemolymph, such as insulin, were not involved in these senescence regulations. Therefore, we examined the relationship between the insulin-signaling activities and the above AstA/Dh31-knockdown phenotypes. We used a reporter gene, tGPH, encoding the GRP1's PH domain fused to GFP, which is localized to the intracellular side of the plasma membrane in response to the activation of PI3K, a transducer of insulin signaling (Brito et al., 2002). In fact, we confirmed that the tGPH protein is localized to the plasma membrane of all types of midgut cells in response to high nutrient supply (data not shown). However, all the AstA/Dh31-knockdown and control flies showed almost the same subcellular localization other than to the plasma membrane (Supplementary Figure S5). These results suggest that senescence control by midgut hormones is less likely mediated by insulin signaling, although a major part of the brain-producing fraction of AstA has been reported to act as a neurotransmitter to affect the production of insulin in the seven pairs of IPCs (Hentze et al., 2015).

Midgut senescence by AstA and Dh31 mediated by authentic senescence mechanisms through JNK, JAK/STAT, and Hippo signaling

The mechanisms for midgut senescence have been extensively studied recently. In a normally aged midgut, the ECs elevate the signaling levels of a stress-responsive protein kinase, c-Jun N-terminal Kinase (JNK), (Biteau et al., 2008). We thus observed JNK activity in the midgut with the senescence phenotype by the AstA receptor-knockdown mosaic. The JNK activity can be monitored by expression of puck-lacZ, an enhancer-trap of the puckered (puc) gene that encodes a MAP kinase phosphatase for negative feedback in the JNK signaling pathway. In the case of AstA-R1 knockdown, although insulin signaling is not considered involved in this process, as described above, JNK activation was obviously observed, similarly to normal senescence. However, unlike normal senescence, in which damage starts from ECs and the unpaired (upd) gene is expressed in early ECs (Biteau et al., 2008; Jiang et al., 2009), senescence by AstA-R1 knockdown seemed to begin from the ISC/EB pairs (ellipses in Supplementary Figure S8A) because the targets of AstA are EBs, as described above. The JNK-active ECs are known to express Upd1, Upd2, and Upd3 proteins, which are matricrine-diffusible ligands in JAK/STAT signaling (Okumura et al., 2014). These ligands show partially redundant functions while showing differential expression in some contexts (Hombria et al., 2005; Yang et al., 2015; Chakrabarti et al., 2016). In the case of this AstA-R1 knockdown, the Upd
family of proteins was similarly induced in JNK-active ISC/EB pairs, but the family member was Upd3, not Upd1 or Upd2 (Supplementary Figure S8B, C). The expressed Upd is known to stimulate the surrounding cells to activate the JNK signal for further expression of the upd3 gene (Buchon et al., 2013; Okumura et al., 2014). Therefore, activation of JNK and Upd3 may gradually expand from the ISCs/EBs that started JNK activation. In fact, the non-cell autonomy of both JNK activation and upd3 expression in ECs was observed (yellow arrowheads in Supplementary Figure S8A', C'). Finally, expression of the lacZ-reporter gene for expanded (ex-lacZ), which is a signature of the repression of proliferation-inhibitory Hippo signaling, was clearly observed in wide areas containing senescence-starting ISCs/EBs (Supplementary Figure S8D). Loss of Hippo signaling is also known to induce upd expression (Shaw et al., 2010). In the areas without senescence-starting ISCs/EBs, ex was not expressed (data not shown). Accordingly, also in the case of the AstA knockdown-mediated midgut, a non-autonomous expansion of cellular senescence phenotypes caused by normal senescence factors could be found, although a family member of Upds and starting cell types showed minor differences from those in normal midgut senescence.

Fig. 6. Premature senescence in male accessory gland by Dh31 knockdown and no effect in Malpighian tubules by AstA or Dh31 knockdowns. (A–F) En face views of male accessory glands (ag) and (G–L) Malpighian tubules in pros-GAL4 (A), (B), (G), and (H), pros > AstA1R (C), (D), (I), and (J), and pros > Dh311R (E), (F), (K), and (L) stained for senescence-associated β-galactosidase activity in blue. Left panels for each organ are derived from 7-day-old adults and right panels are from 28-day-old adults. In the male accessory gland, the number of β-galactosidase-positive cells increased in the 28-day-old pros > Dh311R fly (F). (F') Close-up view of β-galactosidase-positive region of male accessory gland epithelium in F. In old flies in any genotypes, ejaculatory ducts (ed) at the base of two accessory gland lobes are hypersensitive to SA-β-galactosidase activities. Scale bars: 500 μm in A and also applicable to (B–E), 500 μm in (G) and (H–L).

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DISCUSSION

Lifespan and senescence control by antagonistic midgut hormones

Through searching for functions of the midgut hormones AstA and Dh31, we found that they regulated midgut senescence as well as lifespan in adult flies. These two hormones are known to control feeding, water absorption, peristaltic activities, and digestive enzyme activities in the intestine in a wide variety of insects (Lange et al., 1993; Duve and Thorpe, 1994; Fuse et al., 1999; Furuya et al., 2000; Audsley et al., 2008) as well as in Drosophila (Veenstra, 2009b; LaJeunesse et al., 2010; Vanderveken and O’Donnell, 2014). Accordingly, it can be assumed that one of the target organs of these intestinal hormones is the intestine itself. Furthermore, the extent of intestinal target cells for these hormones have not been studied. In the present study, through receptor knockdown experiments, we demonstrated that AstA-R1 indeed acted on the midgut epithelium and further revealed that the target cells for senescence control were EBs. Although it was assumed that midgut senescence started from injured ECs (Amcheslavsky et al., 2009; Jiang et al., 2009; Okumura et al., 2016), we found that the EBs without receiving AstA could be a primary cause of midgut senescence. Also, Dh31 seemed to act as a factor antagonistic to AstA, as previously shown in diuretic activity and contraction regulations (Vanderveken and O’Donnell, 2014). Thus, we expected that these two hormones would have a similar antagonistic relationship in various physiological aspects in addition to these activities. In fact, we clearly found an antagonism between these hormones in controls of midgut senescence and individual longevity. The normal ISC proliferation rate might also be regulated similarly by these hormones (Fig. 7), although the effect of AstA is not obvious at present (Fig. 3D).

Opposed effect on senescence between organs

One of the interesting findings was that Dh31 knockdown repressed senescence in the midgut but induced it in the male accessory gland. Why do both organs show opposed phenotypes in senescence progression? It might be related to the difference in energy consumption and its trade-off relationship between these organs. The midgut and accessory glands are the two largest internal organs that may compete for investment of systemic energy. When Dh31 is knocked down, water absorption of the midgut is less activated than that in the wild type (Vanderveken and O’Donnell, 2014) (Supplementary Figure S7D), which causes lower concentration of digested food and slower energy consumption. To adapt to such conditions, each EB intends to produce and accumulate as much varied secretory peptides as possible. This is because a larger amount of accessory-gland secretion is known to lead to higher male reproductive success (Wigby et al., 2009). Accordingly, to increase male reproductive success, an excess energy supply by lower gut activity leads to higher cell activity in the accessory gland, which results in acceleration of its senescence. As a result, the same hormone Dh31 may provoke different responses between these organs. With regard to sensitivities in AstA action on organ senescence control, we argue that similar differences exist between organs, though our present data only partially support this hypothesis.

Significance in separating AstA and Dh31 into distinct EE subtypes

Even in a single subtype of EE, several types of peptide hormones are produced at the same time. However, AstA and Dh31 are always produced in different EE subtypes. Why are AstA and Dh31 produced separately? As described above, these two hormones have contrasting functions with regard to diuretic action control (Vanderveken and O’Donnell, 2014). If these two hormones are produced in the same cells, their release to activate or inactivate water absorption should thus be controlled by the regulatory secretion pathway rather than the constitutive secretion pathway, to avoid simultaneous release of both hormones. However, another plausible and more reasonable way to differentially secrete these hormones is the production of each hormone in different EE subtypes with different patterns of cell distribution in accordance with the local requirement for each EE subtype. Because the main functions of the PMG are nutrient absorption, activation and inactivation of water absorption should...
be respectively required around the entrance and exit of the PMG to temporarily retain the high concentration of digested food inside the PMG lumen. In fact, the frequencies in each EE subtype vary among positions along the anteroposterior axis (Fig. 1, Supplementary Figures S1, S2), where the AstA subtype is enriched in the posterior region, while the Dh31 subtype is enriched in the anterior region. Thus, this might be the reason AstA and Dh31 are not produced in the same cell. For this requirement, these two subtypes of cell populations create complementarily distributed double gradients in the PMG.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

KTak carried out most of the experiments. TO cooperated for the senescence analyses. MT measured the longevity of GATAe-knockdown flies. MY observed distribution of normal EE subtypes. KTak made the updr3-RedStinger transgenic fly and advised on various experiments. TAY organized the project wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online (URL: http://www.bioone.org/doi/suppl/10.2108/zs160210).

Supplementary Figure S1. Complementary gradient distribution of AstA and Dh31 subtypes of EEs in adult PMG.

Supplementary Figure S2. Wide area view of adult midgut EEs.

Supplementary Figure S3. No significant reduction in AstA and Dh31 levels in brain of midgut-preferential knockdown flies.

Supplementary Figure S4. Shortening of adult lifespan in GATAe-knockdown flies with highly reduced midgut.

Supplementary Figure S5. Localization of tGPH in AstA- and Dh31-knockdown flies.

Supplementary Figure S6. Midgut Dh31 is epistatic to AstA in senescence regulation.

Supplementary Figure S7. Less water-absorption phenotype of midgut by Dh31 knockdown.

Supplementary Figure S8. Knockdown of AstA-Receptors non-autonomously activated JNK and JAK/STAT signaling and inhibited Hippo signaling in PMG.

Supplementary Table S1. List of the fly genotypes used in this study.

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Hormones control senescence and lifespan

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