TITLE:

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AUTHOR(S):

Watanabe, Kaori; Furumizo, Yuki; Usui, Tadao; Hattori, Yukako; Uemura, Tadashi

CITATION:

Watanabe, Kaori ...[et al]. Nutrient-dependent increased dendritic arborization of somatosensory neurons. *Genes to Cells* 2017, 22(1): 105-114

ISSUE DATE:

2017-01-12

URL:

<http://hdl.handle.net/2433/230352>

RIGHT:

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Nutrient-dependent increased dendritic arborization of somatosensory neurons

Kaori Watanabe^{1,2}, Yuki Furumizo^{1,2}, Tadao Usui¹, Yukako Hattori^{1,3}, and Tadashi Uemura^{1,3}

¹ Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan

² These authors contributed equally to this work

³ To whom correspondence should be addressed

Short title: Nutrient-dependent dendrite shaping

Authors for correspondence: Tadashi Uemura and Yukako Hattori

Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan

(Phone) +81-75-753-9238

(Fax) +81-75-753-4265

(E-mail) tauemura@lif.kyoto-u.ac.jp

yhattori@lif.kyoto-u.ac.jp

Keywords: nutrition; neuron; dendrite; arborization; multidendritic neuron;

Drosophila

Abstract

Suboptimal nutrition imposes developmental constraints on infant animals, which marshal adaptive responses to eventually become mature adults. Such responses are mounted at multiple levels from systemic to cellular. At the cellular level, the underlying mechanisms of cell proliferation control have been intensively studied. However, less is known about how growth of postmitotic and morphologically complex cells, such as neurons, is controlled by nutritional status. We address this question using Class I and IV dendritic arborization neurons in *Drosophila* larvae. Class IV neurons have been shown to sense nociceptive thermal, mechanical, and light stimuli, whereas Class I neurons are proprioceptors. We reared larvae on diets with different protein and carbohydrate content throughout larval stages, and examined how morphologies of Class I or IV neurons were affected. Dendritic arbors of Class IV neurons became more complex when larvae were reared on a low-yeast diet, which contains lower amounts of amino acids and other ingredients, compared to a high-yeast diet. In contrast, such low yeast-dependent hyper-arborization was not seen in Class I neurons. The physiological and metabolic implications of the hyper-arborization phenotype are discussed in relation to a recent hypothesis that Class IV neurons sense protein-deficient stress and to our characterization of how the dietary yeast contents impacted larval metabolism.

Introduction

Unlike well-controlled laboratory conditions, development of newborn animals in nature is challenged by a number of changeable environmental conditions, for which they have evolved numerous adaptive responses. One such critical condition is nutritional status, which has profound effects on animal development (Andersen et al., 2013). Rearing model animals under various dietary conditions has been a powerful strategy to unravel evolutionarily conserved adaptive mechanisms. *Drosophila melanogaster* has emerged as an experimental paradigm, owing to its similarities to mammals, including segmented digestive tracts for nutrient intake, hormones and their signaling pathways, nutrient breakdown and storage, and effects of nutrients on lifespan and reproduction (Baker and Thummel, 2007; Lemaitre and Miguel-Aliaga, 2013; Padmanabha and Baker, 2014; Lee et al., 2008; Solon-Biet et al., 2014).

Some of the effects of dietary constraints are manifested by life-history traits at the organism level or by cell behaviors in organogenesis. For example, in the face of nutritional restriction, the juvenile phase is extended to compensate for the slow increase in body size, causing a delay in the transition from juvenile-to-adult (Danielsen et al., 2013). Another example is the systemic control of cell proliferation in many organs, which coordinates downsizing of those organs and the body when nutrients are scarce (Anderson et al., 2013).

In contrast to proliferative cells, such as neural stem cells (Lanet

and Maurange, 2014), relatively little is known about how growth of post-mitotic and morphologically complex cells is influenced by nutritional status, and whether observed nutrient-dependent changes are physiologically relevant or not. Recent studies that address this question include investigations of tracheal terminal cells that innervate the midgut (Linneweber et al., 2014), a subset of serotonergic neurons that innervate the prothoracic gland (Shimada-niwa and Niwa, 2014), and a family of *Drosophila* somatosensory neurons, dendritic arborization (da) neurons (Shimono et al., 2014). da neurons elaborate their dendritic arbors two-dimensionally underneath the epidermis. They are classified into 4 morphological categories, Classes I-IV, in order of increasing territory size and/or branching complexity at the mature larval stage (Grueber et al., 2002; Jan and Jan, 2010). Class IV neurons are polymodal nociceptors responsible for thermal, mechanical, and light sensation (Tracey et al., 2003; Hwang et al., 2007; Xiang et al., 2010; Zhong et al., 2010; Im and Galko, 2012; Terada et al., 2016). Intriguingly, it was recently proposed that Class IV neurons also sense nutrient stress (Jayakumar et al., 2016). On the other hand, Class I neurons function in a proprioceptive sensory feedback circuit for rhythmic locomotion (Hughes and Thomas, 2007; Hwang et al., 2007; Im and Galko, 2012).

In this study, we reared larvae on different diets throughout larval stages and examined how morphologies of Class I or IV neurons were affected. Dendritic arbors of Class IV neurons became more complicated when larvae were reared on a low-yeast diet. In contrast, nutrient-dependent hyper-arborization was not seen in Class I neurons.

Results and Discussion

Dendrites of Class IV neurons hyper-arborized on a low-yeast diet

In a previous study, a severe reduction in dietary yeast from 8% to 0.8% decreased branching of tracheal terminal cells (Linneweber et al., 2014). In the larval diet, yeast is the major source of amino acids, although it also contributes other dietary components (Broderick and Lemaitre, 2012; Piper et al., 2013). When larvae were fed a corn meal-based diet (Table S1), we confirmed the decrease in tracheal branching on the 0.8% yeast (Y) diet compared to the 8% Y diet. In sharp contrast to the tracheal branching, however, dendritic arbors of Class IV neurons became more complicated when larvae were reared on the 0.8% Y diet (Figure 1A and 1B). Quantitatively, both the number of branch ends and the branch density (terminal number/arbor size) were increased by 1.6-fold and 1.7-fold, respectively (Figure 1E-1G), which we refer to hereafter as the hyper-arborization response to the low-yeast diet.

We could reproduce this hyper-arborization phenotype in both sexes when larvae were fed on diets prepared following a different recipe, semi-defined medium (SDM)-based diets with different amounts of yeast-derived ingredients (Figure 1C, 1D, and 1H-1J; see details in Table S1). Again, the number of branch terminals per cell increased by 1.6-fold (males) and 1.4-fold (females) on 0.8% Y compared to 8% Y (Figure 1H). The average number of branch ends on the 2% Y diet was intermediate between those observed with 0.8% Y and 8% Y (Figure S1), reinforcing the notion that the hyper-

arborization is dependent on the decrease in the dietary yeast content. This yeast content-dependent hyper-arborization phenotype was detected using four different transgenic markers consisting of eGFP or tagged fluorescent proteins driven by two different promoters (Figure S2).

Regarding the scale of the arbor, on the SDM-based diets, the arbor size was dramatically larger on 8% Y than 0.8% Y (Figure 1I; increases by 1.7-fold in males and 1.6-fold in females), which is correlated with the larger body size on 8% Y, and this provides a sharp contrast with the relatively lower branch density on 8% Y (Figure 1J). These results raise the possibility that dendritic branching of Class IV neurons is regulated by a mechanism partly separate from the control of arbor size, which is consistent with our previous finding in a different developmental context (Shimono et al., 2014).

The entire larval development took longer (ca. 13 days) on SDM (0.8% Y) compared to 5 days on SDM (8% Y). This raises a question that the hyper-arborization could be a secondary consequence of the longer larval stage. To address this possibility, we observed Class IV neurons in larvae that had been reared on a SDM-based low sugar diet (LSD) or on a high sugar diet (HSD) that elicits insulin-resistant phenotypes (Musselman et al., 2011). It is known that HSD extends larval development; however, we found no drastic increase in the terminal number on HSD compared to LSD (Figure S3). Thus, we consider it less likely that the hyper-arborization was a simple consequence of the longer larval stage.

In addition to the radially expansive and complicated dendrites of Class IV neurons, we examined how the smaller and simpler comb-like arbors of

proprioceptive Class I neurons were affected by the dietary yeast content. Responses of Class I arbors were distinct from those of Class IV (Figure 2). Class I dendrites did not become hyper-arborized on 0.8% Y compared to 8% (Figure 2C-2E) or even became simplified as shown by the decrease in the number of branch terminals by 26% on 0.8% Y (Figure 2F-2H). Together with the aforementioned results for Class IV neurons, these results imply that the two classes of da neurons possess distinct regulatory mechanisms of nutrient-dependent branch sprouting, possibly due to the distinct physiological roles (see below).

A number of cell-autonomous programs have been discovered that control morphogenesis of dendritic arbors of da neurons (Jan and Jan, 2010; Hattori et al., 2013; Iyer et al., 2013; Santiago and Bashaw, 2014; Honjo et al., 2016). Implicated proteins in da neurons include Pathetic (Path), most likely responsible for intake of amino acids (Lin et al., 2015a and 2015b), and insulin receptor and its downstream components (Parrish et al., 2009; Shimono et al., 2014), all of which are required for expansive growth and the highly elaborated architecture of Class IV arbors. The low-yeast dependent hyper-arborization is also reminiscent of an overexpression phenotype of insulin growth factor II mRNA binding protein (Imp) (see Fig 5A, 5C, and 5I in Hattori et al., 2013). However, only a few studies have directly addressed whether or not da neurons detect external nutrient levels. Physiologically, Class IV neurons sense thermal, mechanical, and short-wavelength light stimuli that cause tissue damage, and elicit stereotyped avoidance behaviors (Tracey et al., 2003; Hwang et al., 2007; Xiang et al., 2010; Zhong et al., 2010; Im and Galko, 2012;

Terada et al., 2016). Furthermore, Class IV neurons control light avoidance upon reception of a neuropeptide, the prothoracicotropic hormone (Yamanaka et al., 2013). While Class IV-mediated avoidance behaviors have been well characterized, Class IV neurons also regulate the transition from foraging (feeding) to wandering (non-feeding) behavior at late larval stages on a standard diet (Ainsley et al., 2008; Wegman et al., 2010). Channels and receptors responsible for these physiological roles have been reported (Adams et al., 1998; Tracey et al., 2003; Lee et al., 2005; Neely et al., 2010 and 2011; Hwang et al., 2012; Zhong et al., 2012; Gorczyca et al., 2014; Guo et al., 2014; Mauthner et al., 2014; Honjo et al., 2016).

Very recently a circuit in the larval nervous system has been reported, which is proposed to sense amino acid deprivation and contribute to the juvenile-to-adult transition (pupariation) under nutrient-sparse conditions (Jayakumar et al., 2016). More specifically, Class IV neurons sense amino acid deprivation at a late larval stage and send inputs to glutamatergic neurons in the CNS, which release peptides to neurosecretory cells to modulate ecdysteroid gene expression. However, in their experiments, activities of Class IV neurons were not directly monitored upon the switch from a normal diet to a protein-deficient (sucrose only) diet or vice versa; nor did they examine dendrite morphologies. In contrast to their experimental design, larvae in our study were chronically exposed to the low-yeast or high-yeast diet. Further work will be required to compare neuronal activities and dendrite morphologies under their and our experimental conditions, and to address whether nutrient-dependent neuronal firing

patterns if detected and reshaping of dendrites, such as the hyper-arborization, could be adaptive responses to the lack of amino acids. It should be mentioned that dietary yeast contributes not only amino acids, but also sterols, vitamins, and other known and unknown ingredients (Broderick and Lemaitre, 2012; Piper et al., 2013; Lee and Micchelli, 2013). Therefore, a degree of caution is called for in comparisons of results obtained from yeast-containing diets with those from yeast-free (sugar only) diets.

Potential systemic signals that connect nutrient sensing tissues and dendrite branching of Class IV neurons

In addition to direct sensing of external nutrients by Class IV neurons, endocrine systems coexist, where some tissues secrete systemic signals in response to specific nutritional status (e.g., changes in amino acid and/or carbohydrate levels), and signal receiving tissues, presumably including Class IV neurons, modulate gene expression (Alfa and Kim, 2016). As an initial step to address this hypothesis, we characterized how the dietary yeast contents in our experiments impacted larval metabolism by measuring basic metabolites in whole-body lysates and hemolymph (Figure 3). Whole body glycogen and protein levels were decreased in larvae on 0.8% Y compared to 8% Y (Figure 3C and 3G), which is consistent with the smaller body size. The amount of glucose plus trehalose (the major circulating sugar in insects) normalized to total protein was increased in 0.8% Y larvae relative to 8% Y larvae, but such an increase was not detected in hemolymph (Figure 3B and 3H). Triglyceride (TAG) is the main form of stored fat, and its normalized level was increased in both

sexes of 0.8% Y larvae relative to 8% Y larvae (Figure 3F). Whether this increase in TAG is causally related to the neuronal phenotype we observed awaits future studies.

In one of the endocrine systems, the fat body (the adipose tissue) senses sufficient nutritious sugar, releases one of the *Drosophila* TGF β /Activin homologs, Dawdle (Daw), and controls expression of digestive enzymes in the midgut (Chng et al., 2014) and cellular metabolic and nuclear-encoded mitochondrial genes in the fat body (Ghosh and O'Connor, 2014). Daw signaling requires expression of isoform C of type I receptor Baboon (Babo-C) on target cells (Jensen et al., 2009). Fat bodies also respond to a low level of dietary yeast, release the TNF homolog Eiger (Egr), and negatively control gene expression of insulin-like peptides in insulin-producing cells (IPCs) through one of the two Egr receptors, Grindelwald (Grnd) (Agrawal et al., 2016). Furthermore, a circulating peptide Stunted (Sun) produced by the fat body is a ligand to Methuselah (Mth) receptor on IPCs to release insulin-like peptides (Delanoue et al., 2016). Class IV neurons might be target cells of fat body-derived Daw, Egr, and/or Sun, which might regulate the branching machinery.

It has been shown that Class IV neurons are indeed targets of Egr that is released from UV-induced apoptotic epidermal cells, which contributes to nociceptive sensitization (Babcock et al., 2009). In this context, another Egr receptor, Wengen (Wgn), is necessary in Class IV to develop the sensitization after the UV treatments (Babcock et al., 2009). All of the receptor genes mentioned above are included in the lists of “bound genes”

or “dependent genes” of key transcription factors controlling Class I and IV sub-type specification (Hattori et al., 2013). In parallel to the expression analysis in Class IV neurons, the endocrine hypothesis would be testable by disrupting the relevant receptors or downstream pathways in Class IV neurons and observing if the hyper-arborization still occurs under the low-yeast diet condition. Finally, the impacts of low dietary protein on lifespan and reproduction are conserved between flies and mice (Lee et al., 2008; Solon-Biet et al., 2014); therefore, the nutrient-dependent hyper-arborization of neurons might also occur in mammals.

Experimental procedures

Fly stocks and the database

Strains employed were *ppk-GAL4 UAS-mCD8:GFP* on the 3rd chromosome (Grueber et al., 2007), *ppk-eGFP* (Grueber et al., 2003a), *GAL4[2-21] UAS-mCD8:GFP* (Grueber et al., 2003b), *Gr28b.c-GAL4 UAS-mCD8:GFP* (Xiang et al., 2010) and *ppk-CD4:tdTom* on the 3rd chromosome (Han et al., 2011). The yeast content-dependent hyper-arborization of Class IV neurons was detected in a total of four different genotypes: *ppk-eGFP/ppk-eGFP* (Figure 1A, 1B, and 1E-1G), *ppk-GAL4 UAS-mCD8:GFP/+* (Figure 1C, 1D, and 1H-1J), *ppk-CD4:tdTom/+* (Figure S2A-E), and *Gr28b.c-GAL4 UAS-mCD8:GFP/+* (Figure S2F and G). Throughout this study, FlyBase was used (Attrill et al., 2016).

Diets and rearing

Our stocks are usually reared on a laboratory standard diet. To observe how dendrite morphologies of da neurons were affected under different nutritional conditions, we cooked corn meal diets or modified semi-defined media (SDM) with different yeast or sugar amounts. Nutritional contents of all of the diets are shown in Table S1. The original semi-defined medium (SDM) is as described at the Bloomington Stock Center (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/germanfood.htm).

Adult males and virgin females that had developed on the standard diet were collected and crossed on the standard diet for three days or for seven days with one transfer to vials of the fresh standard diet in mid-course. Then the adults

were split into two or three groups, each of which was kept on the corn meal-based diet or SDM-based diets with different yeast amounts (see details in the legend of Table S1). After an egg-laying interval of one-to-two days, the adult flies were cleared in every experiment. Wandering 3rd instar larvae that came out of individual diets were imaged or used to prepare lysates as described below. All the crosses and experiments were conducted under non-crowded conditions at 25°C.

Imaging and quantification

Images of da neurons in A3-A5 segments were acquired and quantitatively analyzed essentially as described (Matsubara et al., 2011; Hattori et al., 2007 and 2013; Parrish et al., 2009).

Measurements of basic metabolites

We essentially followed Tennessen et al. (2014) and Matsuda et al. (2015) to measure amounts of basic metabolites in whole-body lysates of larvae. Each lysate was made from two larvae. To measure trehalose (the primary circulating sugar in hemolymph) and glycogen, we enzymatically digested the lysates to release free glucose by using Trehalase (SIGMA T8778) and Amyloglucosidase (SIGMA A1602), respectively. We used a Glucose (HK) Assay Kit (SIGMA GAHK-20), Triglyceride E-Test Wako (Wako 432-40201), and DC Protein Assay (Bio-Rad 500-0116) to measure glucose, triglyceride (TAG), and protein, respectively. Absorbance was read according to the manufacturer's protocol using a Tecan GENious Microplate Reader. Endogenous levels of free glucose in larval lysates

were low and sometimes undetectable, so we added values of glucose to those of trehalose. Hemolymph was prepared from five larvae per sample as described by Matsuda et al. (2015).

Acknowledgements

The reagents, genomic datasets, protocols, and/or facilities were provided by the Kyoto Stock Center (DGRC), the Bloomington Stock Center, Vienna *Drosophila* Resource Center, the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947), the NIG stock center, FlyBase, modENCODE, Y.N. Jan, S. Hirabayashi, M. Negishi, H. Kato, I. Oinuma, and T. Nishimura. We also thank K. Oki and M. Futamata for their technical assistance, other Uemura lab members for critical discussion, and J. Hejna for polishing the manuscript. This work was supported by a grant from Grants-in-Aid for Scientific Research (A) (15H02400 to T. Uemura) of MEXT, a grant from the Mitsubishi Foundation to T. Uemura, a Grant-in-Aid for Young Scientists (B) (15K18455 to Y. H.) of MEXT, grants from the Naito Foundation and the Sasakawa Scientific Research Grant to Y. H., and the Platform Project for Supporting in Drug Discovery and Life Science Research (Platform for Dynamic Approaches to Living System) from Japan Agency for Medical Research and Development (AMED). K.W. is a recipient of a JSPS Research Fellowship for Young Scientists.

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Figure legends

Figure 1 Dendritic arborization of Class IV neurons of larvae grown on different diets.

(A-D) Representative images of dendritic arbors of Class IV neuron ddaC. In this and subsequent figures, anterior is left and dorsal is up, and images of the dorsal cluster in wandering 3rd instar stage are shown. The magenta arrow in A indicates the cell body of ddaC. (A, B, and E-G) Corn meal-based diets. (C, D, and H-J) SDM-based diets. The yeast content was 8% Y (A and C) or 0.8% Y (B and D). (E-J) Quantitative analyses. The numbers of branch terminals (E and H), the size of the dendrite arbor (F and I), and the terminal number divided by the arbor size (G and J). n=9 except for 0.8% Y males in H-J (n=7). **: P<0.01; ***: P<0.001 (Wilcoxon-Mann-Whitney test). Scale bars: 200 μ m. Relevant genotypes in A, B, and E-G are *ppk-eGFP/ppk-eGFP* and those in C, D, and H-J are *ppk-Gal4 UAS-mCD8:GFP/+*.

Figure 2 Dendritic arborization of Class I neurons when larvae were fed on different diets

(A and B) Representative images of dendritic arbors of Class I neurons ddaD and ddaE. The magenta arrows indicate cell bodies of ddaD (left) and ddaE (right). (C-H) Quantitative analyses. The numbers of branch terminals (C and F), the size of the arbor (D and G), and the terminal number divided by the arbor size (E and H). n=9 (8% Y) and 6 (0.8% Y). *: P<0.05; **: P<0.01 (Wilcoxon-Mann-Whitney test). Scale bars: 200 μ m. The relevant genotype is

GAL4[2-21] UAS-mCD8:GFP/+.

Figure 3 Carbohydrates, TAG, and protein content of larvae grown on 8% Y or 0.8% Y diets

Larvae grown on SDM containing 8% Y or 0.8% Y were sorted by sex and processed for whole lysates (A-G) or hemolymph (H). Concentrations of glucose plus trehalose (A), glycogen (C), TAG (E), and protein (G) in larval lysates, and the respective values normalized to total protein (B, D, and F). (H) The concentration of glucose plus trehalose in hemolymph. n=8-10.*: P<0.05; **: P<0.01; ***: P<0.001 (Wilcoxon-Mann-Whitney test). The relevant genotype is *Gr28bc-GAL4 UAS-mCD8:GFP/+*.