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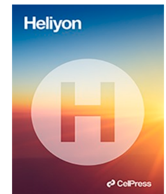

Research article

Molecular and cellular organization of odorant binding protein genes in *Drosophila*

Keehyun Park [a,1], Hyungjun Choi [a,1], I Joon Han [b,1], Wayessa Rahel Asefa [a,1], Chaiyoung Jeong [a], Seungyun Yu [a], Hanhee Jeong [a], Minkook Choi [c], Sung-Eun Yoon [d], Young-Joon Kim [d,e], Min Sung Choi [a,**], Jae Young Kwon [a,*]

[a] Department of Biological Sciences, Sungkyunkwan University, Suwon, 16419, Republic of Korea
[b] Sungkyunkwan University School of Medicine, Seoul, 06351, Republic of Korea
[c] Department of Oral Biology, Yonsei University College of Dentistry, Seoul, 03722, Republic of Korea
[d] Korea Drosophila Resource Center, Gwangju, 61005, Republic of Korea
[e] School of Life Sciences, Gwangju Institute of Science and Technology (GIST), Gwangju, 61005, Republic of Korea

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ABSTRACT

Chemosensation is important for the survival and reproduction of animals. The odorant binding proteins (OBPs) are thought to be involved in chemosensation together with chemosensory receptors. While OBPs were initially considered to deliver hydrophobic odorants to olfactory receptors in the aqueous lymph solution, recent studies suggest more complex roles in various organs. Here, we use *GAL4* transgenes to systematically analyze the expression patterns of all 52 members of the *Obp* gene family and 3 related chemosensory protein genes in adult *Drosophila*, focusing on chemosensory organs such as the antenna, maxillary palp, pharynx, and labellum, and other organs such as the brain, ventral nerve cord, leg, wing, and intestine. The OBPs were observed to express in diverse organs and in multiple cell types, suggesting that these proteins can indeed carry out diverse functional roles. Also, we constructed 10 labellar-expressing *Obp* mutants, and obtained behavioral evidence that these OBPs may be involved in bitter sensing. The resources we constructed should be useful for future *Drosophila* OBP gene family research.

1. Introduction

Detection of the surrounding environment to induce an appropriate response is essential to animal survival and reproduction. In particular, chemosensation, such as olfaction and gustation, is mainly utilized in behaviors such as finding food, selecting a mate, and avoiding predators, among many other functions. Following identification of various gene families in the *Drosophila* genome involved in detecting chemicals in the olfactory and gustatory organs, such as the odorant receptors (OR), gustatory receptors (GR), ionotropic receptors (IR), and odorant binding proteins (OBP) [1–5], research mainly focused on the various receptors, while relatively few studies focused on the OBPs. OBPs are compact, soluble proteins thought to act in olfaction by aiding in the transport of volatile chemicals within the sensory organs of both vertebrates and insects, where they are secreted. Due to their stable structure, OBPs are

* Corresponding author.
** Corresponding author.
E-mail addresses: minsung.choi@gmail.com (M.S. Choi), jykwon@skku.edu (J.Y. Kwon).
[1] These authors contributed equally.

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being investigated for potential engineering applications across various systems including biosensors, ligand binding assays, anchorage systems, analytical tools, and repellent design [6].

There are 52 OBPs in *D melanogaster* that share only 20% amino acid identity but have a very well conserved structure: small size (about 14 kDa) and a globular formation, a cavity for ligand binding, and six cysteines with conserved spacing to form disulfide bonds [7–10]. Regarding the functions of *Drosophila* OBPs, a few examples demonstrate roles in olfaction. According to previous studies [11–13],OBP76a, commonly known as Lush, binds *cis*-Vaccenyl acetate (cVA), a male-specific sexual pheromone, and transfers it to Or67d, which mediates aggregation behavior in fruit flies. OBP28a mediates the perception of floral odorants and acts as a buffer in situations with varying odor concentrations [14]. RNAi-mediated suppression of *Obp* gene expression led to changes in behavioral responses to multiple odorants, with effects varying depending on sex [15]. In *Drosophila*, several OBPs are highly expressed in olfactory tissues such as the antennae and maxillary palps [16,17], and thus a widely held assumption was that the OBPs would bind hydrophobic odorants in the aqueous sensillar lymph and help transport the odorants across the lymph to their receptors in the dendritic membranes of olfactory receptor neurons. However, recent functional data demonstrating robust olfactory responses in the absence of abundant antennal OBPs suggested the need to take a broader view of the roles of OBPs, and that their tasks may not be limited to olfaction or even chemosensation [18,19].

A few studies suggest a gustatory role for certain OBPs. OBP49a, which is expressed in the thecogen cells, accessory cells of the sensory neurons in the sensillum, acts in the inhibition of sweet sensing neurons by bitter chemicals [20]. OBP19b has been implicated in amino acid sensation [21]. Furthermore, RNAi-induced decrease of the expression of individual *Obp* genes led to either increased or decreased consumption of sucrose in the presence of bitter compounds [22].

Several examples exist of OBPs with atypical roles independent of chemosensation. OBP56g is crucial to male fertility, with mutant males lacking OBP56g failing to induce the formation of a mating plug in the female reproductive tract, leading to ejaculate loss and reduced sperm storage [23]. OBP59a is expressed in the sacculus, a hygrosensory location of the antennae [17]. OBP50d has been connected to resistance to starvation, and OBP50a has been associated to the determination of sex ratios [24]. As can be seen in these studies from *Drosophila* and other species, OBPs act in diverse tissues and organs, including those with non-chemosensory functions, suggesting diverse roles for these proteins beyond chemosensation [8].

*Drosophila*odorant binding proteins (OBPs) were initially described in olfactory sensilla [25], and initial studies on the OBPs mainly focused on the olfactory system among the chemosensory systems. A subset of *Drosophila* OBPs were previously reported to be expressed in taste appendages [5,7,20,21,26], and these proteins were recently discovered to express in other chemosensory and non-chemosensory organs [8]. However, a systematic characterization of the expression of all members of the *Obp* gene family has not yet been attempted.

Here we describe a set of transgenic reporters for the entire *Obp* repertoire. We use these to survey the expression of this gene family in various tissues and organs in the adult fly. By examining *GAL4*-driven GFP reporter expression, we observed the expression patterns of all 52 members of the *Obp* gene family and 3 related chemosensory protein genes in the olfactory organs (antenna and maxillary palp), and organs with gustatory neurons (labellum, pharynx, leg, and wing), and the brain, ventral nerve cord, and intestine. The *Obp-GAL4* drivers are expressed in diverse cell types and organs, and expression was not limited to chemosensory function-related cells, with some expressing in mechanosensory neurons. We especially focused on *Obp-GAL4* drivers that express in the labellum, the main taste organ in the fly head, to categorize expression by cell types such as accessory cells including tormogen and thecogen cells, as well as glia, gustatory receptor neurons, and epidermal cells. Also, behavioral experiments using mutants suggested that some of these *Obp* genes are involved in bitter sensing.

2. Results

2.1. A toolkit of transgenic reporters for OBPs

To systematically examine the expression of the entire repertoire of odorant binding proteins (OBPs) in *Drosophila*, we used the *GAL4-UAS* system. We generated over 500 *Obp-GAL4* transgenic lines for 47 *Obp* genes and 2 related chemosensory protein genes, *Os-C* and *Os-D/antennal protein 10*. In addition to these 49 genes, we additionally analyzed *GAL4* transgene expression of 5 *Obp* genes and one chemosensory protein gene with already available transgenes (*Obp19b, Obp19d, Obp49a, Obp56g, Obp59a,* and *CheB42a*), for a total of 55 genes. For convenience, in the remainder of the paper, we will refer to the 52 *Obp-GAL4* and 3 *chemosensory protein-GAL4* drivers collectively as the 55 *Obp-GAL4* drivers. For the *GAL4* drivers we generated, as many as 10 independent lines were examined for each driver in an effort to accurately determine the expression pattern of each gene. For certain previously published *Obp* transgenes, a single line was analyzed. In total, we analyzed 241 lines for the 55 *Obp-GAL4* drivers, a mean of 4.4 lines/driver. We examined adult flies heterozygous for each *GAL4* driver with two copies of the *UAS-mCD8-GFP* reporter. For each OBP or chemosensory protein, the expression pattern observed in the majority of lines was defined as the representative pattern. Among the lines showing the representative pattern, one or two lines that showed the most consistent and penetrant expression was selected as the representative line for each OBP or chemosensory protein.

2.2. Obp-GAL4 transgenes are expressed in diverse organs

To examine *Obp-GAL4* transgene expression using a GFP reporter, the second antennal segment, third antennal segment, maxillary palp, labellum, pharynx, leg, wing, brain, ventral nerve cord, and intestine were dissected, stained and observed (Fig. 1). Among the 55 *Obp-GAL4* transgenes, 42 were expressed in diverse patterns, with some expressed specifically in one organ and some broadly in many

organs, while 13 were not expressed in any of the organs we observed (Fig. 1).

Consistent with the OBPs having initially been found in the olfactory appendages, 22 and 13 of the 55 *Obp-GAL4* drivers were observed in the third segment of the antenna and maxillary palp, respectively, which are olfactory organs of the adult fly. In a previous study, the transcriptome of the third segment of the *Drosophila* antenna was obtained by mRNA sequencing (RNA-Seq), and antennal expression of 27 of the 52 members of the OBP family was identified [16]. Among these genes, transcript expression of the 10 *Obps* most abundantly expressed in the antenna was verified using *in situ* hybridization [19]. We also observed expression of these 10 *Obps*

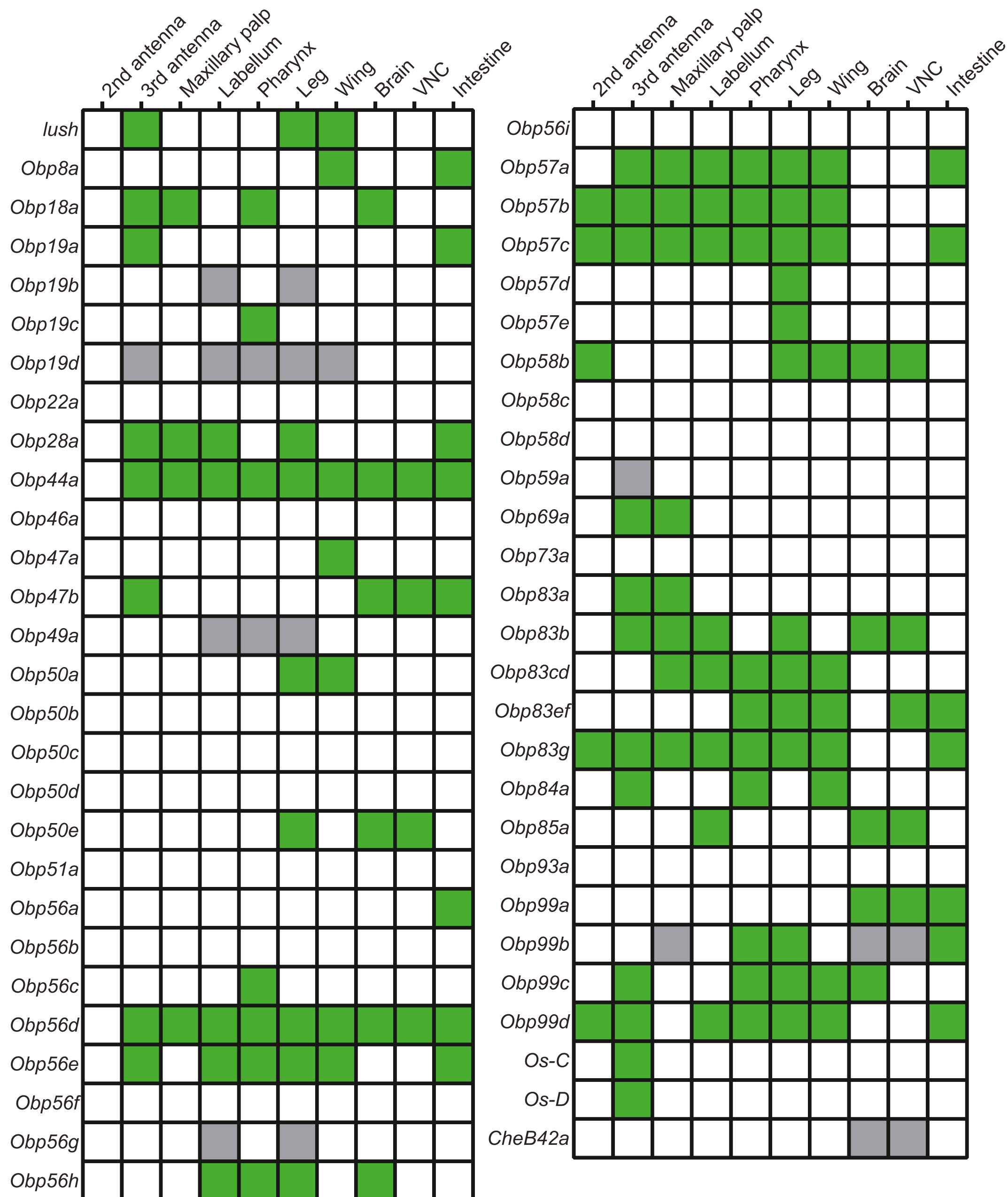

Fig. 1. Expression catalog for *Obp* and related chemosensory genes. Expression patterns of 52 *Obp* and 3 related chemosensory genes (*Os-C*, *Os-D*, and *CheB42a*) were investigated in various adult peripheral chemosensory organs (second segment of antenna, third segment of antenna, maxillary palp, labellum, pharynx, leg, and wing), as well as the brain, ventral nerve cord (VNC), and intestine. Deep green shading highlights show where expression was detected with newly generated *Obp-GAL4* drivers. Conversely, grey shading indicates expression patterns observed with independently generated, previously reported *Obp-GAL4* drivers. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(*Obp19a, Obp19d, Obp28a, Obp56d, Obp59a, Obp69a, Obp76a/lush, Obp83a, Obp83b,* and *Obp84a*) in the third segment of the antenna using *GAL4* transgene expression (Fig. 1). Among the 17 *Obps* observed to have a lower level of expression by RNA-Seq, we observed the GFP expression of 8 *Obp-GAL4* (*Obp18a-, Obp44a-, Obp56e-, Obp57a-, Obp57b-, Obp57c-, Obp83g-,* and *Obp99c-GAL4*) lines in the antenna (Fig. 1). Additionally, two *Obp* genes, *Obp47b* and *Obp99d*, that were not expressed at a significant level in the antenna by RNA-Seq, were observed to show *GAL4* transgene expression in the antenna (Fig. 1). *GAL4* transgenes of two of the chemosensory protein genes that we examined, *Os-C* and *Os-D/a10*, were observed to specifically drive GFP reporter expression in the third antennal segment, and not in any other tissues examined (Fig. 1). Overall, in the third antennal segment, expression of the *Obp* genes as observed by RNA-Seq, *in situ* hybridization, and *GAL4* transgenes appears to agree fairly well, providing some assurance of the accuracy of representation of actual *Obp* gene expression of these *Obp-GAL4* lines in other tissues as well.

14 *Obp-GAL4* drivers were observed to express GFP in the central nervous system (brain and/or ventral nerve cord) (Fig. 1). Among these, certain drivers with expression in labellar gustatory receptor neurons (GRNs) that show axonal projections in the subesophageal zone (SEZ), such as *Obp83b-* and *Obp85a-GAL4* (detailed explanation below), were also included in the count of GFP expression in the CNS, although in this case the *Obp-GAL4* may not actually be expressed in cells of the CNS. Among the *Obp-GAL4* drivers expressed in the CNS, *Obp44a-* and *Obp99a-GAL4* stood out because of their potential expression in glial cells (Fig. 2A). The *Drosophila* blood-brain barrier is composed of perineurial and subperineurial glial cells [27], and these two *Obp-GAL4* drivers show a blood-brain barrier (BBB)-like pattern. This expression pattern is consistent with a transcriptomic study of *Drosophila* surface glia, in which one of the top 50 most abundantly expressed genes was *Obp44a* [28]. *Obp44a-GAL4* is also expressed in many peripheral cells including cells of the antenna, labellum, maxillary palp, pharynx, and wings (Fig. 1). This expression pattern suggests *Obp44a* expression in wrapping glial cells, which wrap and support sensory and motor axons in the *Drosophila* peripheral nervous system (Fig. 2A) [29].

The legs were dissected and examined for each sex, as morphological features of the fly legs are sexually dimorphic, but none of the *GAL4* drivers showed sexually dimorphic expression patterns except for *lush* (*Obp76a-GAL4*), which showed weaker expression signals in the female leg. A total of 25 *Obp-GAL4* drivers appeared to express in the legs, and among these, three *Obp-GAL4* drivers (*Obp50e-*, *Obp56d-*, and *Obp58b-GAL4*) showed GFP reporter expression in the femoral chordotonal organ (FeCO) (Fig. 2B). The FeCO is the largest organ related to proprioception in fruit flies [30]. These three *Obp-GAL4* drivers also showed ventral nerve cord projection patterns similar to proprioceptor-related neurons (Fig. 2B). *Obp58b-GAL4* was also observed to express in the Johnston's organ in the second segment of antenna, which has a role in the fly auditory system [31,32], and *Obp56d-GAL4* was expressed in the arista of the antenna, as previously observed [19], suggesting that OBPs may also be involved in mechanosensory perception (Fig. S1).

In addition, 19, 18, and 15 *Obp-GAL4* drivers were observed to drive GFP reporter expression in the pharynx, wing, and gut, respectively, although some expression may be due to non-specific ectopic expression of transgenes. A more detailed analysis of the cell types that show expression and the functional relevance of expression is a task we leave for future studies. In summary, as examined through *GAL4* transgene expression, the *Obp* gene family members are expressed in a diverse spectrum of tissues and organs.

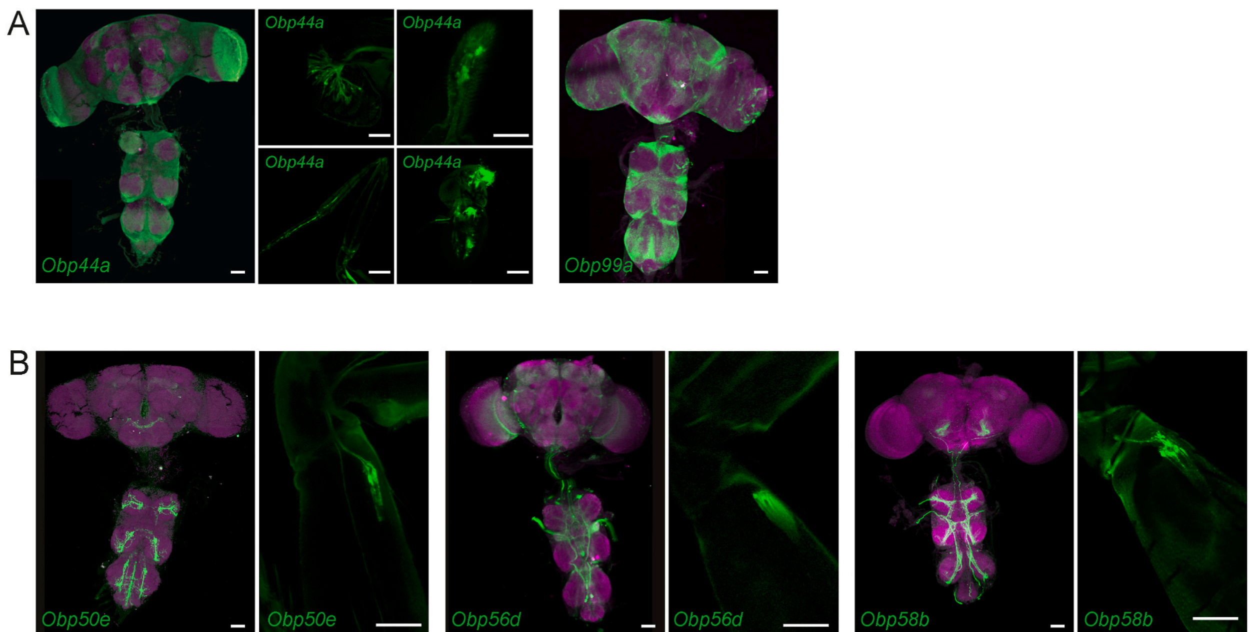

Fig. 2. *Obp-GAL4* drivers expressed in glia or mechanosensory organs. (A) *Obp44a-* and *Obp99a-GAL4* drivers showed glial cell-like expression patterns. *Obp44a-GAL4* also expressed in the labellum (upper left), maxillary palp (upper right), leg (lower left), and antenna (lower right). (B) Expression patterns of *Obp50e-*, *Obp56d-*, and *Obp58b-GAL4* drivers in the CNS and femoral chordotonal organ (FeCO). Scale bar = 50 μm.

2.3. Obp-GAL4 transgenes expressed in the labellum

Drosophila taste sensilla are located in the labellum, pharynx, anterior margin of the wing, tarsi, and female genitalia [33,34]. To identify OBPs that function in gustation, we examined *Obp-GAL4* drivers that express in the labellum, the major taste organ of the adult head, in greater detail. The *Drosophila* labellum has sensilla which are composed of sensory neurons, supporting cells, and glial cells wrapping the sensory neurons' axons (Fig. 4A). We observed that 17 *Obp-GAL4* drivers expressed in the labellum (Figs. 1 and 3), including the previously reported *Obp19b* [21], with expression in diverse cell types such as the sensory neurons, glia, gland, supporting cells, and some cells that were difficult to identify by gross anatomy (Fig. 3).

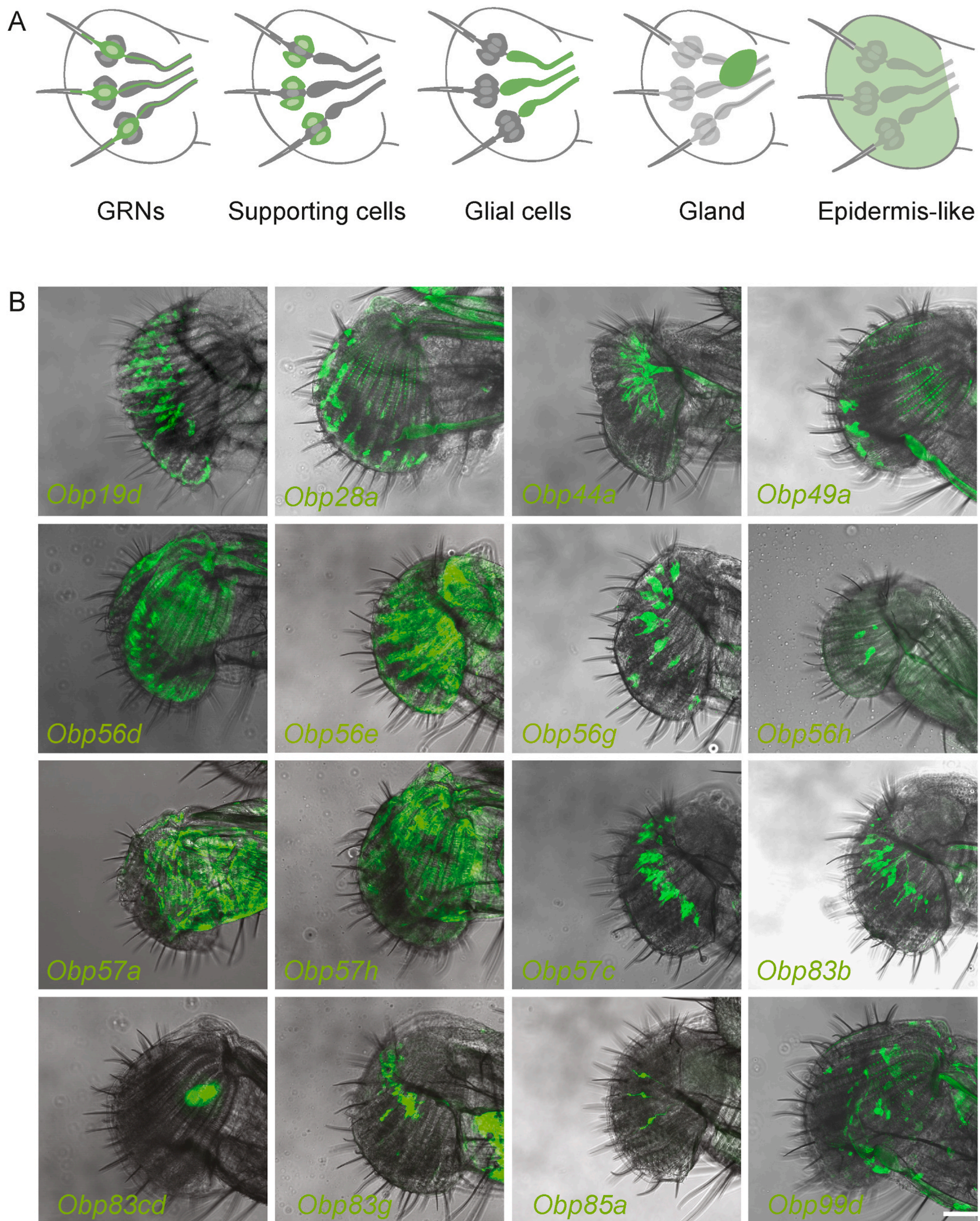

Fig. 3. *Obp-GAL4* drivers expressed in the labellum. (A) Schematic drawings describing 5 types of cells in fly labellum. The number and shape of each cell type are simplified. GRN; gustatory receptor neuron. (B) Labellums immunostained for GFP expressed by 16 *Obp-GAL4* drivers. Scale bar = 50 μm.

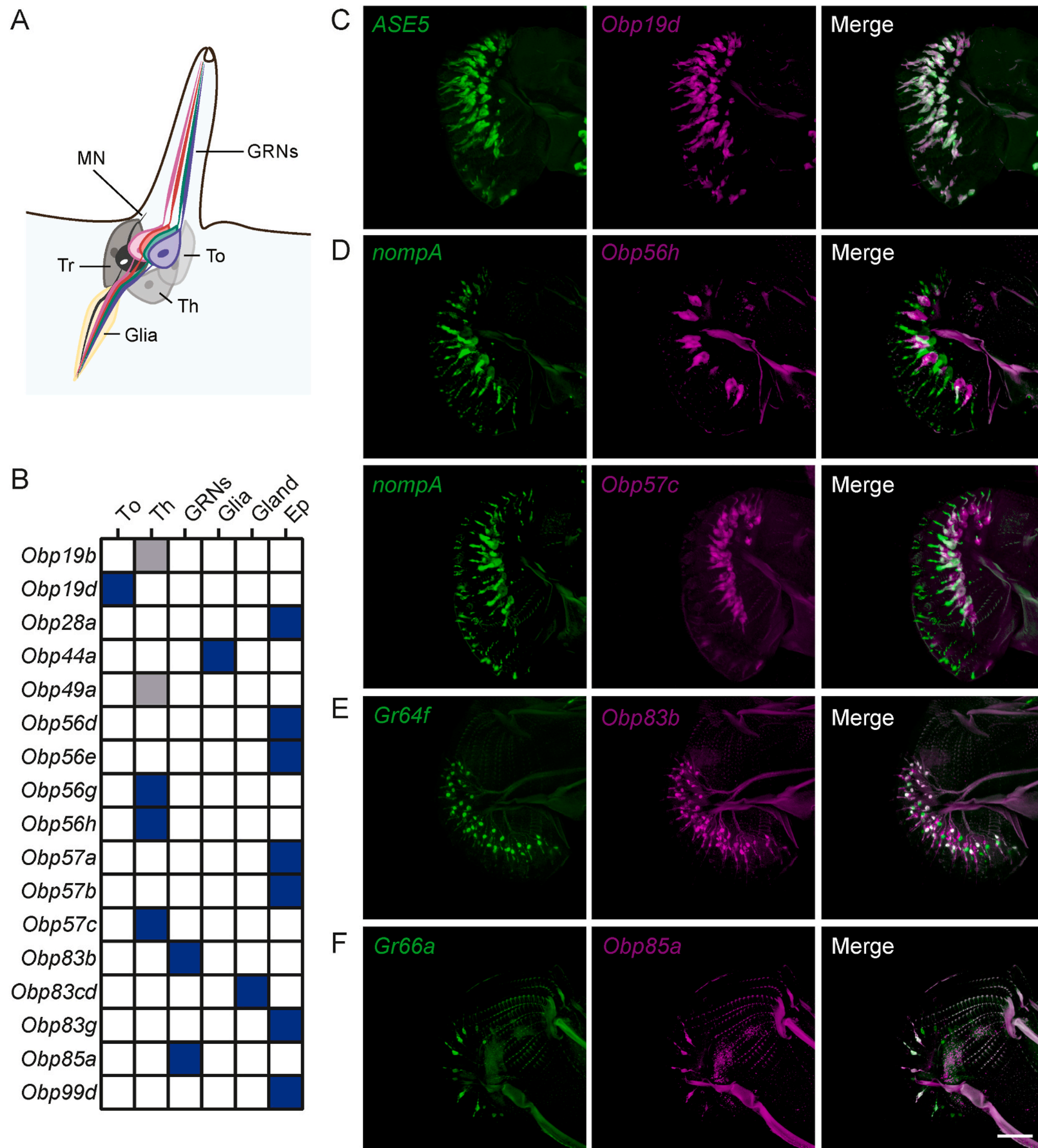

Fig. 4. *Obp-GAL4* drivers expressed in labellar accessory cells and gustatory receptor neurons. (A) Schematic drawing describing a labellar taste sensillum containing sensory neurons, glia, and accessory cells. A mechanosensory neuron (MN) and four gustatory receptor neurons (GRNs) are surrounded by glia and three supporting cells: the tormogen (To), thecogen (Th), and trichogen (Tr) cells. (B) Summary of 17 *Obp-GAL4* drivers expressed in the labellum classified by the identified cell types. Deep blue shading highlights where expression was detected with newly generated *Obp-GAL4* drivers. Conversely, grey shading indicates expression patterns observed with independently generated *Obp-GAL4* drivers, which were previously reported. Ep: Epidermis-like cell. (C) Co-labeling of tormogen (*ASE5*) and *Obp19d-GAL4* driver. (D) Co-labeling of thecogen (nompA) and *Obp56h-* and *Obp57c-GAL4* drivers. (E) Co-labeling of *Gr64f-lexA*-expressing GRNs and *Obp83b-GAL4* driver. (F) Co-labeling of *Gr66a-lexA*-expressing GRNs and *Obp85a-GAL4* driver. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

To identify the positions and types of the cells where each *Obp-GAL4* transgene was expressed, we used the *UAS-RedStinger/UAS-myr.GFP* reporter, which marks the nucleus with RFP and intracellular membrane weakly with GFP (Fig. S2). 7 *Obp-GAL4* drivers (*Obp28a-*, *Obp56d-*, *Obp56e-*, *Obp57a-*, *Obp57b-*, *Obp83g-*, and *Obp99d-GAL4*) appeared to express in what are likely epidermal cells (Fig. 3), although we cannot completely rule out the possibility of expression in the endocuticle. *Obp83*cd-*GAL4* appeared to express in gland cells (Fig. S2).

The supporting cells are tormogen, thecogen, and trichogen cells [35]. The functions of those supporting cells are not clearly known yet, but the OBPs are generally considered to be expressed in and secreted from these cells. To identify the *Obp-GAL4* drivers expressed in the supporting cells, we first double-labeled the cells with *ASE5-GFP* which labels tormogen cells, and observed that among the

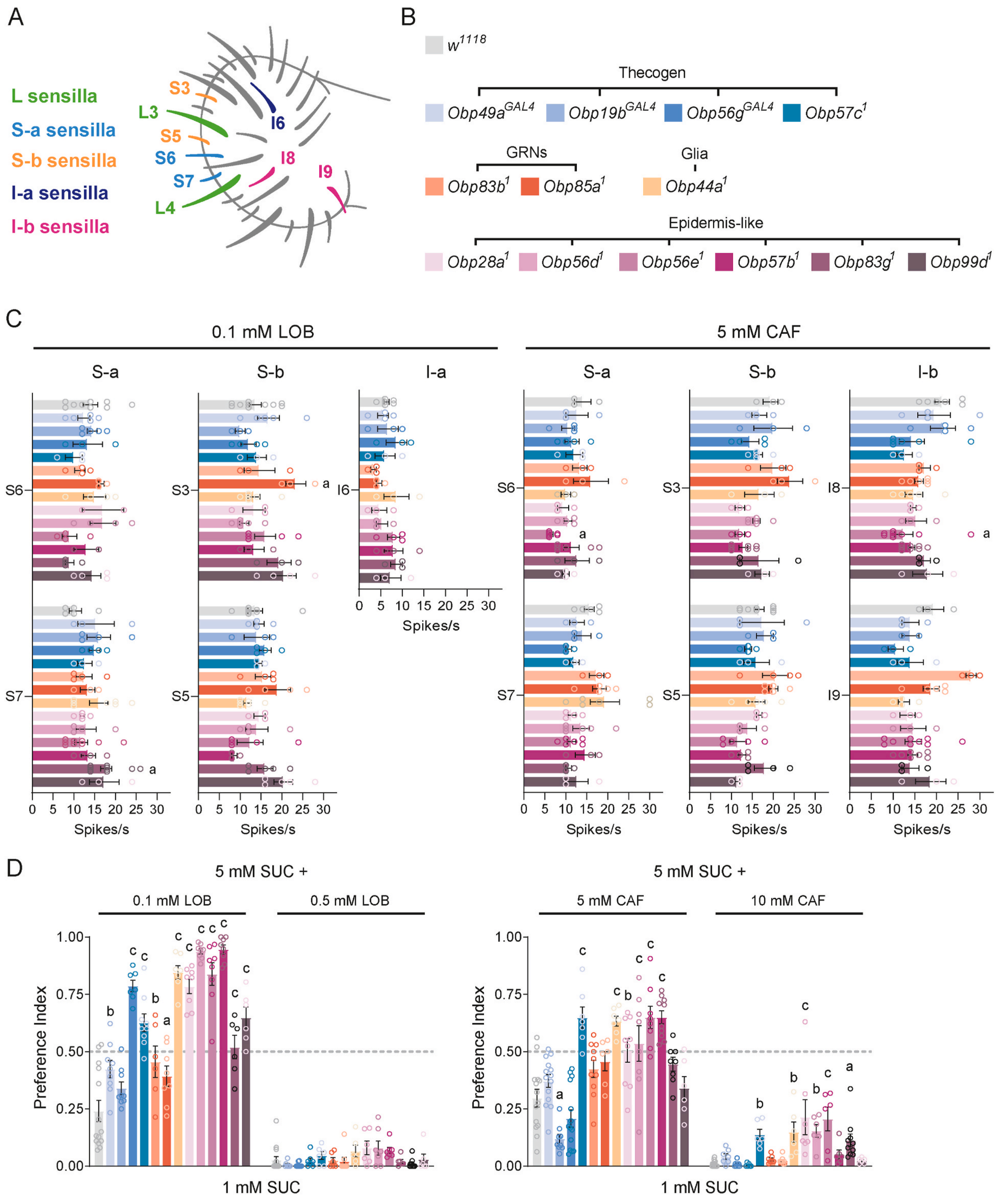

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Fig. 5. Taste responses of labellum-expressed *Obp* gene mutants. (A) Schematic drawing describing a typical fly labellum and the types of taste sensilla. L3 and L4 belong to L-type sensilla (green), S6 and S7 to S-a type sensilla (light blue), S3 and S5 to S-b type sensilla (orange), I6 to I-a sensilla (deep blue), and I8 and I9 to I-b type sensilla (magenta). (B) Color code of w^{1118} and *Obp* gene mutants for Fig. 5C and D. (C) Neuronal taste responses to 0.1 mM lobeline (left) and 5 mM caffeine (right). In the case of I-type sensilla, I6 sensillum (I-a type) was tested for lobeline and I8 and I9 sensilla (I-b type) were tested for caffeine, respectively. n = 2–10. (D) Behavioral avoidance responses to caffeine (left) and lobeline (right) at the indicated concentrations. n = 6–14 Ordinary one-way ANOVA followed by post hoc Dunnett's multiple comparison test. a: $p < 0.05$, b: $p < 0.005$, c: $p < 0.001$. Statistical analysis compared the mean of each group with the mean of control w^{1118} (light grey). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Obp-GAL4 drivers expressed in the labellum, only *Obp19d-GAL4* was co-labeled with *ASE5-GFP* (Fig. 4B, C and S3). Previous studies showed that OBP49a and OBP19b are expressed in thecogen cells, the accessory cells surrounding the base of taste sensory neurons found in labellar sensilla [20,21]. We observed that *Obp56h-* and *Obp57c-GAL4* expressing cells co-labeled with *nompA-GFP*, a thecogen cell marker (Fig. 4C). *Obp56g-GAL4* expression became very weak when combined with *nompA-GFP*, hindering observation of co-localization, but *Obp56g-GAL4* expressing cells were directly adjacent to *ASE5-GFP* expressing tormogen cells, suggesting expression in the thecogen cells (Fig. S3A). These results suggested that at least 5 OBPs are expressed in the thecogen cells.

Obp44a-, *Obp83b-*, and *Obp85a-GAL4* are expressed in cells that are definitely distinguishable from tormogen or thecogen cells (Fig. S3B). As mentioned above, *Obp44a-GAL4* appears to be expressed in glial cells, which ensheath the axons of sensory neurons (Fig. S3B) [36]. *Obp83b-* and *Obp85a-GAL4* showed neuronal expression, and so we attempted double-labeling with drivers that label GRNs. *Obp83b-GAL4* expression in the labellum overlapped to a large degree with *Gr64f-lexA* expression, which label the sugar sensing neurons, but expression did not completely overlap, and either expression was not a subset of the other (*Gr64f* + *Obp83b* + 33 cells ± 2.52, *Gr64f* + *Obp83b-* 5.3 cells ± 2.73, *Gr64f- Obp83b* + 25 cells ± 3.51, mean ± SEM, n = 3) (Fig. 4E). Also, expression of *Gr64f-lexA* expressing neurons and *Obp83b-GAL4* expressing neurons did not overlap in the legs (Fig. S4). *Obp85a-GAL4* expression in the labellum was observed to be a subset of *Gr66a-lexA* expressing neurons, which specifically label the bitter GRNs (*Gr66a* + *Obp85a*+ 3.8 cells ± 0.37, *Gr66a* + *Obp85a-* 8 cells ± 0.55, *Gr66a- Obp85a*+ 0, mean ± SEM, n = 5) (Fig. 4F), and considering the number of neurons and the position of the labellar sensilla, appear to be expressed in the S-b sensilla (Fig. S5) [37]. The axon projection patterns of the subesophageal zone of the *Drosophila* brain, which receives taste sensory input, were also consistent with OBP83b mainly expressing in sweet neurons, and OBP85a expressing in bitter neurons (Fig. S6).

2.4. Functional analysis of OBP mutants suggests involvement in bitter sensing

To examine whether the OBPs we observed to express in the labellum were involved in taste perception, we constructed *Obp* mutants. Among the 17 *Obp* genes whose expression we observed in the labellum using *GAL4* transgenes, mutants were already available for *Obp19b*, *Obp49a*, and *Obp56g*, and thus we attempted to construct mutants for the remaining 14 *Obp* genes. Deletion mutants for 10 *Obp* genes were successfully obtained through the CRISPR-Cas9 method (details in Methods), including mutants for *Obp57c* (thecogen cells), *Obp83b* (mainly sugar neurons), *Obp85a* (bitter neurons), *Obp44a* (glia), *Obp28a*, *Obp56d*, *Obp56e*, *Obp57b*, *Obp83g*, and *Obp99d* (likely epidermis). We examined the electrophysiological responses of the 13 *Obp* mutants to a sweet substance (sucrose) and bitter substances (lobeline and caffeine).

The labellum is comprised of 31 taste sensilla categorized into three classes based on their length: long (L-type), short (S-type), and intermediate (I-type) (Fig. 5A) [33,38,39]. Sugar neurons in L sensilla elicit robust electrophysiological responses to diverse sugar compounds [40]. None of the *Obp* mutants we tested showed a significant change in response to sucrose in the L3 and L4 sensilla compared to the control w^{1118} line (Fig. S7A). The labellar sensilla can be clustered into five functional classes on the basis of response spectra, with S-a and S-b responding to a broad range of bitter compounds, and I-a and I-b specifically responding to lobeline and caffeine, respectively [37]. The 13 *Obp* mutants did not show a significant difference in bitter response profile from w^{1118} (Fig. 5C). When we examined the behavioral responses of the 13 *Obp* mutants, all of the mutants showed a clear preference for sucrose (Fig. S7B). In our experiments, we were not able to observe the clear counteraction of 5 mM caffeine to the sugar-driven attractive response observed in a previous study [20], but we did observe that avoidance was reduced in response to low concentrations of bitter substances (0.1 mM lobeline, 5 mM caffeine) (Fig. 5D). In summary, although we were not able to identify labellar-expressing *Obp* mutants that showed robust impairment in electrophysiological response to sucrose, lobeline, or caffeine, our behavioral experiments suggest the possibility that OBPs may influence bitter sensing as previously reported [22].

3. Discussion

In this study, we used *GAL4* transgene-driven GFP reporter expression to examine and identify the expression of 52 members of the *Obp* gene family and 3 related chemosensory protein genes in various organs of the fruit fly. Recently, reports regarding the expression and function of *Obp* genes in various organs, not limited to the olfactory organs, have been increasing [8], and our study provides support for those studies as well as valuable resources for future OBP studies.

We observed that 17 *Obp-GAL4* drivers show expression in the labellum, the mouth of the fly. These *Obp-GAL4* drivers were expressed in the tormogen cells (*Obp19d*), thecogen cells (*Obp19b*, *Obp49a*, *Obp56g*, *Obp56h*, and *Obp57c*), gustatory receptor neurons (*Obp83b* and *Obp85a*), glia (*Obp44a*), glands (*Obp*83cd), and epidermis-like cells (*Obp28a*, *Obp56d*, *Obp56e*, *Obp57a*, *Obp57b*, *Obp83g*, and *Obp99d*). The expression patterns of 10 *Obp* genes expressed at the highest levels in the third segment of the antennae, the main *Drosophila* olfactory organ, were studied in detail to identify cell types showing expression [19], and among these, 4 genes were also

identified to express in the labellum (Fig. 1). *Obp28a* was identified to express in the tormogen and thecogen cells of the antenna [19] and the epidermal cells of the labellum (Fig. 4B), while *Obp83b*, which expresses in the antennal tormogen cells [19], also expresses in the sweet-sensing neurons of the labellum (Fig. 4B). *Obp19d* and *Obp56d*, which express in the antennal epidermal cells [19], were observed to express in the tormogen and epidermal cells of the labellum, respectively (Fig. 4B). When *Obp-GAL4* drivers are expressed in the epidermis of the labellum, it may be difficult to identify expression of the drivers in supporting cells such as the thecogen cells located inside the epidermis, due to the high expression levels in the epidermis. Also, due to the lack of a marker for trichogen cells, we may have missed *Obp-GAL4* expression in trichogen cells. It was indeed suggested that most of the *Obp* probes expressed in the antenna seem likely to be expressed in trichogen cells [19]. Overall, the OBPs appear to be expressed in the supporting cells (tormogen, thecogen, trichogen cells) or epidermal cells of sensory receptor neurons in different sensory organs, and the specific cell type that shows expression appears to vary from gene to gene.

In some cases, such as *Obp83b-GAL4*, *Obp* genes show a much more complicated expression pattern than expected. The *Obp83b* gene is located less than 1 kb from *Obp83a* and encodes a protein with 68% amino acid identity to OBP83a [41], and the two *Obp* genes show an almost identical expression pattern, with expression in the tormogen cells of the same set of basiconic sensilla [19]. Through our *GAL4* drivers, *Obp83a-GAL4* was expressed only in the olfactory organs (antenna and maxillary palp) and not in other organs, while *Obp83b-GAL4* was expressed in the antenna as well as taste sensory neurons of the labellum and legs. What was striking was that while many *Obp83b-GAL4* expressing neurons overlapped with *Gr64f-lexA* expressing neurons, which label the sugar sensing neurons (Fig. 4E), no overlap was observed in the legs (Fig. S4). This may suggest that *Obp83b-GAL4* is expressed in a subset of attractive taste neurons that detect a functionally distinctive orphan taste class. Of course, due to the caveats of transgene expression, we cannot rule out the possibility of ectopic expression, and additional experiments need to be conducted to confirm the functional relevance of this expression. Although with the same caveats, some *Obp-GAL4* drivers showed expression that warrant further interest. The *Drosophila* glial cells are classified into a few subtypes: cortex glia, surface glia, astrocyte-like glia, ensheathing glia (also called wrapping glia in the PNS), perineurial glia, and subperineurial glia [27,42,43]. *Obp44a*- and *Obp99a-GAL4* display expression consistent with the morphology of the *Drosophila* blood-brain barrier (BBB), which is composed of perineurial and subperineurial glial cells and is a primary immune barrier between the central nervous system and hemolymph. *Obp44a-GAL4* also shows a glial cell expression pattern in the peripheral sensory system. Although this is as yet speculation, this expression in the glial cells may suggest the possibility of involvement of the OBPs in the function of *Drosophila* glial cells, such as lipid metabolism, speed regulation of neuronal signaling, or hormonal transportation [27,44–46]. In *C. elegans* and *Drosophila*, an active role of glia has been reported in the olfactory sensory system [47,48], and as such, future studies unraveling the potential influence of glial OBPs on the chemosensory neurons could provide interesting information. *Obp50e*-, *Obp56d*-, and *Obp58b-GAL4* were observed to express in the femoral chordotonal organ (FeCO), Johnston's organ, and arista, suggesting an important role in mechanosensation. In this study, we were not able to analyze expression of the 15 *Obp-GAL4* drivers expressed in the gut in detail, to see if expression was specific to certain regions of the gut, such as the foregut, midgut, or hindgut, or specific to certain cell types, such as the intestinal stem cells, enterocytes, enteroendocrine cells, or muscle cells. The chemosensory function of OBPs in the gut could also be a potentially interesting topic for exploration. We could not detect the expression of 13 *Obp-GAL4* drivers. This could be due to highly specific expression in just a few cells, expression in tissues that were not examined, or expression at different developmental stages.

In this study, we were able to provide experimental evidence suggesting divergent functions of the OBPs in diverse organs, and the resources we provide in this study should be useful for future studies exploring OBP function.

3.1. *Limitations of the study*

Since we used *GAL4* transgenes as the main method to examine *Obp* gene expression, the inherent caveats of our study are that we cannot be sure if the promoter regions used carry sufficient regulatory elements, if all cells and organs where an OBP is expressed were identified through GFP expression, and if the expression observed is not ectopic expression. Also, the method we used would show us the cells that express OBP, but since the OBPs may be secreted to function, accurate assessment of the sites of action would require studies using antibodies. In addition, since we examined the expression of 55 genes, we were not able to examine expression in every organ of *Drosophila* in detail. Our *GAL4* drivers could be used in future studies to examine expression at different developmental stages, such as the larval stage, or in organs such as the reproductive organs. Also, we newly constructed mutants for 10 *Obp* genes, and measured the electrophysiological responses of a limited number of sensilla to limited concentrations of only the most basic tastants (sucrose, lobeline, and caffeine). For a more thorough and accurate examination of the potential functions of the *Obp* genes, experiments should be conducted with an accurate control considering the genetic background, with a much broader panel of chemicals in various contexts.

4. Materials and methods

4.1. *Key resources table*

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-nc82	DSHB	AB_2314866

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse anti-GFP	Invitrogen	A-11120
Rabbit anti-GFP	Invitrogen	A-11122
Chicken anti-GFP	Invitrogen	A-10262
Rabbit anti-RFP	Chemicon international	AB3216
Goat anti-mouse Alexa 488	Invitrogen	A-11001
Goat anti-mouse Alexa 586	Invitrogen	A-11004
Goat anti-rabbit Alexa 488	Invitrogen	A-11008
Goat anti-rabbit Alexa 568	Invitrogen	A-11011
Goat anti-chicken Alexa 405	Invitrogen	A-48260
Chemicals, peptides, and recombinant proteins		
20× Phosphate-buffered saline (PBS)	Biosesang	PR2007-100-00
8% Paraformaldehyde in 2× PBS	Biosesang	PC2184-050-00
Normal goat serum	Jackson immunoresearch	005-000-121
Triton-X	Sigma-Aldrich	X100-500 ML
Sucrose	Duchefa Biochemie	50809.5000
Caffeine	Sigma-Aldrich	C0750
Lobeline hydrochloride	TCI	L0096
Tricholine citrate	Sigma-Aldrich	T0252
Experimental models: Organisms/strains		
D. melanogaster: w^{1118}		N/A
D. melanogaster: lush-GAL4	This study	N/A
D. melanogaster: Obp8a-GAL4	This study	N/A
D. melanogaster: Obp18a-GAL4	This study	N/A
D. melanogaster: Obp19a-GAL4	This study	N/A
D. melanogaster: Obp19b^{GAL4}	Bloomington Drosophila Stock Center	RRID:BDSC_55034
D. melanogaster: Obp19c-GAL4	This study	N/A
D. melanogaster: Obp19d-GAL4	This study	N/A
D. melanogaster: Obp22a-GAL4	This study	N/A
D. melanogaster: Obp28a-GAL4	This study	N/A
D. melanogaster: Obp44a-GAL4	This study	N/A
D. melanogaster: Obp46a-GAL4	This study	N/A
D. melanogaster: Obp47a-GAL4	This study	N/A
D. melanogaster: Obp47b-GAL4	This study	N/A
D. melanogaster: $Obp49a^{GAL4}$	Bloomington Drosophila Stock Center	RRID:BDSC_55033
D. melanogaster: Obp50a-GAL4	This study	N/A
D. melanogaster: Obp50b-GAL4	This study	N/A
D. melanogaster: Obp50c-GAL4	This study	N/A
D. melanogaster: Obp50d-GAL4	This study	N/A
D. melanogaster: Obp50e-GAL4	This study	N/A
D. melanogaster: Obp51a-GAL4	This study	N/A
D. melanogaster: Obp56a-GAL4	This study	N/A
D. melanogaster: Obp56b-GAL4	This study	N/A
D. melanogaster: Obp56c-GAL4	This study	N/A
D. melanogaster: Obp56d-GAL4	This study	N/A
D. melanogaster: Obp56e-GAL4	This study	N/A
D. melanogaster: Obp56f-GAL4	This study	N/A
D. melanogaster: Obp56g^{GAL4}	Bloomington Drosophila Stock Center	RRID:BDSC_55079
D. melanogaster: Obp56h-GAL4	This study	N/A
D. melanogaster: Obp56i-GAL4	This study	N/A
D. melanogaster: Obp57a-GAL4	This study	N/A
D. melanogaster: Obp57b-GAL4	This study	N/A
D. melanogaster: Obp57c-GAL4	This study	N/A
D. melanogaster: Obp57d-GAL4	This study	N/A
D. melanogaster: Obp57e-GAL4	This study	N/A
D. melanogaster: Obp58b-GAL4	This study	N/A
D. melanogaster: Obp58c-GAL4	This study	N/A
D. melanogaster: Obp58d-GAL4	This study	N/A
D. melanogaster: Obp59a-GAL4	Bloomington Drosophila Stock Center	RRID:BDSC_80682
D. melanogaster: Obp69a-GAL4	This study	N/A
D. melanogaster: Obp73a-GAL4	This study	N/A
D. melanogaster: Obp83a-GAL4	This study	N/A
D. melanogaster: Obp83b-GAL4	This study	N/A
*D. melanogaster: Obp83*cd*-GAL4*	This study	N/A
D. melanogaster: Obp83ef-GAL4	This study	N/A
D. melanogaster: Obp83g-GAL4	This study	N/A
D. melanogaster: Obp84a-GAL4	This study	N/A
D. melanogaster: Obp85a-GAL4	This study	N/A
D. melanogaster: Obp93a-GAL4	This study	N/A
D. melanogaster: Obp99a-GAL4	This study	N/A

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(*continued*)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
D. melanogaster: Obp99b-GAL4	This study, Bloomington Drosophila Stock Center	RRID:BDSC_77561
D. melanogaster: Obp99c-GAL4	This study	N/A
D. melanogaster: Obp99d-GAL4	This study	N/A
D. melanogaster: CheB42a-GAL4	Bloomington Drosophila Stock Center	RRID:BDSC_66677, RRID:BDSC_66678
D. melanogaster: Os-C-GAL4	This study	N/A
D. melanogaster: Os-D-GAL4	This study	N/A
D. melanogaster: $Obp28a^{KO}$	This study	N/A
D. melanogaster: $Obp44a^{KO}$	This study	N/A
D. melanogaster: $Obp56d^{KO}$	This study	N/A
D. melanogaster: $Obp56e^{KO}$	This study	N/A
D. melanogaster: $Obp57b^{KO}$	This study	N/A
D. melanogaster: $Obp57c^{KO}$	This study	N/A
D. melanogaster: $Obp58b^{KO}$	This study	N/A
D. melanogaster: $Obp83b^{KO}$	This study	N/A
D. melanogaster: $Obp83g^{KO}$	This study	N/A
D. melanogaster: $Obp85a^{KO}$	This study	N/A
D. melanogaster: $Obp99a^{KO}$	This study	N/A
D. melanogaster: $Obp99d^{KO}$	This study	N/A
D. melanogaster: UAS-redstinger;UAS-GFP	Bloomington Drosophila Stock Center	RRID:BDSC_90912
D. melanogaster: UAS-GFP	Bloomington Drosophila Stock Center	RRID:BDSC_1521, BDSC_1522
D. melanogaster: UAS-RFP	Bloomington Drosophila Stock Center	RRID:BDSC_32223
D. melanogaster: ASE5-GFP	Bloomington Drosophila Stock Center	RRID:BDSC_58449
D. melanogaster: nompA-GFP	Bloomington Drosophila Stock Center	RRID:BDSC_42694
D. melanogaster: $Gr64f^{lexA}$	Bloomington Drosophila Stock Center	RRID:BDSC_93445
D. melanogaster: Gr66a-lexA	Bloomington Drosophila Stock Center	RRID:BDSC_93024
D. melanogaster: UAS-RFP,lexAop-GFP	Bloomington Drosophila Stock Center	RRID:BDSC_32229
Software and algorithm		
Prism 9	GraphPad	https://www.graphpad.com/
LabChart 8	ADinstruments	https://www.adinstruments.com/
ImageJ	Schneider et al. [49]	https://imagej.net/ij/
Adobe Illustrator	Adobe	https://www.adobe.com

4.2. Fly maintenance and transgenic flies

Flies were maintained in standard cornmeal agar medium at room temperature. Genomic DNA of *wCS* or *CS* was used as template to construct transgenic flies. *Obp-GAL4* transgenes and *Obp* mutations were maintained by using *FM7a* (X chromosome), *CyO* (2nd chromosome), and *TM3, Sb* (3rd chromosome) as balancers.

To generate *Obp-GAL4* transgenic flies, the transgenic constructs were created by PCR by amplifying the immediate upstream region of the initiation codon of each *Obp* gene. The Table 1 below describes the primers used to target the upstream region of each OBP gene. The transgenic constructs were injected into w^{1118} embryos using P-element random insertion. The emerging adult flies were crossed with a balancer stock and PCR was used to verify the *GAL4* sequence. For each OBP gene, at least one independent *Obp-GAL4* line per chromosome (X, 2nd, and 3rd) was examined, if possible.

Table 1
Primer sets for the constructs of *Obp-GAL4* drivers.

Gene	Primers (5′-3′)	
Obp76a (lush)	fwd	GAGGTACCTGAGTTATTTTCGCGAGTGC
	rev	GAGCGGCCGCGGTGGCGGAAAGCTAAATACG
Obp8a	fwd	GAGGTACCAAATGCAATGCGACGACGAG
	rev	GAGCGGCCGCTCCCACGGCGATGTCATCGG
Obp18a	fwd	GAGGTACCAATGGGAATTCGCATAGACGA
	rev	GAGCGGCCGCTTGGGAAACAGATTTTCGCC
Obp19a	fwd	GAGGTACCACCGGGATTCGGATTGTGAC
	rev	GAGCGGCCGCTTCCGAGACGATTTGGCGGA
Obp19c	fwd	GAGAATTCGATGCGACATTTCGATTTGC
	rev	GAGCGGCCGCCTTGGCAGCTGCTTTCTCTTTG
Obp22a	fwd	GAGGTACCTAGAGCCTCGACCTATACCG
	rev	GAGCGGCCGCCTCGAAGATCGTTTTTG
Obp28a	fwd	GAGGTACCCCCATCTAAGCGTATAACGC
	rev	GAGCGGCCGCGATGCTAGGTCGGTGTGTC
Obp44a	fwd	GAGGTACCCAGGCGGCTTCTCATGTTG
	rev	GAGCGGCCGCGGTGAATCTTATTTTTTTG

(*continued on next page*)

Table 1 (*continued*)

Gene	Primers (5′-3′)	
Obp46a	fwd	GAGGTACCGTGCCGCTTGATTGCTCTT
	rev	GAGCGGCCGCTTTCCGCAATGCCAGG
Obp47a	fwd	GAGGTACCCCTCGTTCCTTCTAGCGTTCC
	rev	GAGCGGCCGCTTTGTTAAATTCGAATGC
Obp47b	fwd	GAGGTACCAACACACCACTTGGCCTAAC
	rev	GAGCGGCCGCAGCTGCAATTGAGTGAG
Obp50a	fwd	GAGGTACCTTCTGGGAGCACCAACTG
	rev	GAGCGGCCGCAACTAAAGCTACAAC
Obp50b	fwd	GAGGTACCCGCAATCTAAACGGCACTTG
	rev	GAGCGGCCGCTTTGAAATTGCCTCCC
Obp50c	fwd	GAGGTACCCTTTGCAGCCCGAAAGGG
	rev	GAGCGGCCGCTTTTTTAGTCAGTCTGAG
Obp50d	fwd	GAGGTACCAACACAGTTGTGGACGGAG
	rev	GAGCGGCCGCTTCGAGTTACTTCTGCTTTAC
Obp50e	fwd	GAGGTACCGCAAGTGGTTTCTGGCACAATG
	rev	GAGCGGCCGCATTTATGGTGTAGTGATTC
Obp51a	fwd	GAGGTACCTAAGTAGGCCGCTTTGGTG
	rev	GAGCGGCCGCTTTGAGAACTACTAACG
Obp56a	fwd	GAGGTACCTCGTCTGTCTCCTTGCGCTTC
	rev	GAGCGGCCGCGTTGAGAAATACTTTGA
Obp56b	fwd	GAGGTACCGGCAGCGACTTTATCGACG
	rev	GAGCGGCCGCCTTTCCAAAGCTACATTC
Obp56c	fwd	GAGGTACCACCTTTGCGAGACTATGTTCC
	rev	GAGCGGCCGCCTAAGAAATATTTTTAAAA
Obp56d	fwd	GAGGTACCTCGCGTGACATTTGCATTTC
	rev	GAGCGGCCGCTTTCGGTAGAGATGTTG
Obp56e	fwd	GAGGTACCAAGGTAGGCATGGGCTTC
	rev	GAGCGGCCGCGATGCTGATCGTAATCTGC
Obp56f	fwd	GAGGTACCTAGATCAGGCTTCCCAAATAGG
	rev	GAGCGGCCGCAATGATAGTTTTGTGTGCAAG
Obp56h	fwd	GAGGTACCCGTTGGCCTTTGTTTCGTCCTG
	rev	GAGCGGCCGCTTTGAGGTATATATTTGTTAAAG
Obp56i	fwd	GAGGTACCTCGGTTTCGGCGGGTTAGAG
	rev	GAGCGGCCGCAACGTATGTATATCCTGTG
Obp57a	fwd	GAGGTACCCGTCCAAAGTATGCAGCGTG
	rev	GAGCGGCCGCTGTTAACTTCAGACTGAACA
Obp57b	fwd	GAGGTACCGTCAGCGTGGTCTCCATACA
	rev	GAGCGGCCGCTGTAGAAATGAAACTAAACA
Obp57c	fwd	GAGGTACCAATTACCGCCGGGGAAG
	rev	GAGCGGCCGCTATCTAACGATTCGCAGAAT
Obp57d	fwd	GAGGTACCCAGATGACTAACCAAAGGCA
	rev	GAGCGGCCGCCAAACTAGTTGAAGATATCA
Obp57e	fwd	GAGGTACCCTACCAAGCTGGCCGTGCTC
	rev	GAGCGGCCGCACTTGCTATATTCCTAGGGA
Obp58b	fwd	GAGGTACCGGCTAAGAGTACGGTTACGG
	rev	GAGCGGCCGCGATGACAGCTTTCCAGCGGT
Obp58c	fwd	GAGGTACCGTGGGCAGTGTAATTCCATC
	rev	GAGCGGCCGCTGCTCCGCCGTTTATACCAA
Obp58d	fwd	GAGGTACCCACTCCTTGGTCTTCGACCA
	rev	GAGCGGCCGCTGCTTTTCGAATGTGATTAA
Obp69a	fwd	GAGGTACCTGGAACTATCTCTTAAGCTAG
	rev	GAGCGGCCGCTTTTGCTTCTCCCCAAAAAT
Obp73a	fwd	GAGGTACCCTTGTCTCACCTGACCACCC
	rev	GAGCGGCCGCGGTTGCGATTAATTGGAGTC
Obp83a	fwd	GAGGTACCGAAGGCTGATCCTGTCCACTAC
	rev	GAGCGGCCGCTTTAATGTGGCTCTTTCCGTTT
Obp83b	fwd	GAGGTACCTCTTTTCGCGGCATAAACTG
	rev	GAGCGGCCGCTTTGAAACTACAATGAATGG
*Obp83*cd	fwd	GAGAATTCGTTTGGCAACTGCTAACTGG
	rev	GAGCGGCCGCTGCTAAACAATTCTCAATAT
Obp83ef	fwd	GAGGTACCTCTGGTGGCCCAGGTCAGAG
	rev	GAGCGGCCGCCTCTCTGCGGGCAATGCACA
Obp83g	fwd	GAGGTACCTACCTCTGGCTTCCGGTCAA
	rev	GAGCGGCCGCTTCTGGCTCGGACGAGGGCT
Obp84a	fwd	GAGGTACCACCAAACGATCTCATGAATTTGAA
	rev	GAGCGGCCGCCTTGAACAAACAAAGTGCTG
Obp85a	fwd	GAGGTACCAACAAAATCCAACCACTTCCCC
	rev	GAGCGGCCGCTTCAGAACTGACTGATGCAT
Obp93a	fwd	GAGGTACCCACACGCTGCTGCTCGTTGT
	rev	GAGCGGCCGCGTTGTGCTTAAAATTTATAA

(*continued on next page*)

Table 1 (*continued*)

Gene	Primers (5′-3′)	
Obp99a	fwd	GAGAATTCTTTCCATGAGCAACATCATC
	rev	GAGCGGCCGCTTTCACTTTCTTTCCACCTA
Obp99b	fwd	GAGGTACCTGTGTTGGCTTTGGGTGATT
	rev	GAGCGGCCGCGCTGATGTATGTTTACCTTG
Obp99c	fwd	GAGGTACCCTTCGCTCTTTGCTCTC
	rev	GAGCGGCCGCATTGAGATATAGTTAGTTATA
Obp99d	fwd	GAGGTACCCGATTTGCGATTTGCGATTT
	rev	GAGCGGCCGCTGCGTTTGCGACAGCTTTAT
Os-C	fwd	GAGGTACCCGTAAGCACGTTAATCTCAAGC
	rev	GAGCGGCCGCTTTGTCAACTTGGTTGGGGC
Os-D	fwd	GAGGTACCGCCCACAGCAAAGTCGAGAG
	rev	GAGCGGCCGCTTTCGAATCGATCGTATGCTG

To generate *Obp* knock-out mutant flies, CRISPR/Cas9 method was used. Guide sequences were introduced into pU6-BbsI-chiRNA. Detailed information about the guide RNA is in the Table 2 below. Each plasmid at 250 ng/μl was injected into CAS-0001 (*$y^2cho^2v^1$; attP40{nos-Cas9}/CyO*) embryos. The emerging adult flies were crossed with a balancer stock and PCR was used to isolate mutants with deletions between the two target sequences, and the break points were verified by sequencing.

Table 2
Primer sets used and deletion sizes for each *Obp* mutant.

MUTANTS	Guide RNA		Size of deletion (bp)
$Obp28a^1$	1st fwd 5′-3′	CTTCGCAGTCTACTCCAATCATTC	381
	1st rev 5′-3′	AAACGAATGATTGGAGTAGACTGC	
	2nd fwd 5′-3′	CTTCGCCTATGGCACTTGCTTCAG	
	2nd rev 5′-3′	AAACCTGAAGCAAGTGCCATAGGC	
$Obp44a^1$	1st fwd 5′-3′	CTTCGCTGTGCGCCCTGCTGGGTC	451
	1st rev 5′-3′	AAACGACCCAGCAGGGCGCACAGC	
	2nd fwd 5′-3′	CTTCGAACTCCTAGTTCTTCTGGA	
	2nd rev 5′-3′	AAACTCCAGAAGAACTAGGAGTTC	
$Obp56d^1$	1st fwd 5′-3′	CTTCGATTGTCCTCTCCGTCATTT	192
	1st rev 5′-3′	AAACAAATGACGGAGAGGACAATC	
	2nd fwd 5′-3′	CTTCGGAGATTTGGCGCTTAGATG	
	2nd rev 5′-3′	AAACCATCTAAGCGCCAAATCTCC	
$Obp56e^1$	1st fwd 5′-3′	CTTCGCGCCCTTGCAGCTCTATCTT	364
	1st rev 5′-3′	AAACAAGATAGAGCTGCAAGGGCGC	
	2nd fwd 5′-3′	CTTCGTCGCACTTGTCGGCTCCCT	
	2nd rev 5′-3′	AAACAGGGAGCCGACAAGTGCGAC	
$Obp57b^1$	1st fwd 5′-3′	CTTCGTTATTGTTCAGCTTGGCCA	396
	1st rev 5′-3′	AAACTGGCCAAGCTGAACAATAAC	
	2nd fwd 5′-3′	CTTCGTGATGTGTGCATTTAAGGC	
	2nd rev 5′-3′	AAACGCCTTAAATGCACACATCAC	
$Obp57c^1$	1st fwd 5′-3′	CTTCGTTAGATAATGCTTAAGCTA	468
	1st rev 5′-3′	AAACTAGCTTAAGCATTATCTAAC	
	2nd fwd 5′-3′	CTTCGAGCGATGAGGTCACCGAAG	
	2nd rev 5′-3′	AAACCTTCGGTGACCTCATCGCTC	
$Obp83b^1$	1st fwd 5′-3′	CTTCGCGCCTTGGTTCCTGGGCAG	475
	1st rev 5′-3′	AAACCTGCCCAGGAACCAAGGCGC	
	2nd fwd 5′-3′	CTTCGATCAGCCTTCTTCCAGCAT	
	2nd rev 5′-3′	AAACATGCTGGAAGAAGGCTGATC	
$Obp83g^1$	1st fwd 5′-3′	CTTCGGCTGCAACGATCAGCAGGA	456
	1st rev 5′-3′	AAACTCCTGCTGATCGTTGCAGCC	
	2nd fwd 5′-3′	CTTCGCTGCCCATCAACCGCCACG	
	2nd rev 5′-3′	AAACCGTGGCGGTTGATGGGCAGC	
$Obp85a^1$	1st fwd 5′-3′	CTTCGCTGGCAGCATTTAATGGAT	234
	1st rev 5′-3′	AAACATCCATTAAATGCTGCCAGC	
	2nd fwd 5′-3′	CTTCGGTTGATGCCTAGGCAAGAT	
	2nd rev 5′-3′	AAACATCTTGCCTAGGCATCAACC	
$Obp99d^1$	1st fwd 5′-3′	CTTCGAGCTGCCTGCTTATTGCGA	331
	1st rev 5′-3′	AAACTCGCAATAAGCAGGCAGCTC	
	2nd fwd 5′-3′	CTTCGCAGTTTGGCCATTGGGTCA	
	2nd rev 5′-3′	AAACTGACCCAATGGCCAAACTGC	

4.3. Immunohistochemistry and imaging

After obtaining multiple *Obp-GAL4* transgenic lines for a certain *Obp* gene, we dissected at least three males and three females from each line to analyze peripheral expression patterns in the second antennal segment, third antennal segment, maxillary palp, labellum, pharynx, wing, and legs. Expression in the legs was examined with distinction between males and females, but other organs were examined without distinction between the sexes. Upon identification of one or two representative lines with the most consistent and penetrant expression, these lines were further dissected to analyze expression in the brain, ventral nerve cord, and intestines. At least three males and three females were analyzed per line, totaling at least six individuals per selected line, and expression was examined without distinction between the sexes. For immunohistochemistry, to allow for sufficient time for expression of the GAL4 driven GFP reporter, 5 to10-day-old female and male flies were used for dissection. After dissection, organs were fixed in PBS-T (phosphate-buffered saline with 0.2% Triton-X, pH7.2) containing 4% formaldehyde for at least 2 h on ice. The fixed organs were washed with PBS-T three times for 15 min each and blocked in 3% normal-goat-serum-containing PBS-T for 2 h. The samples were then stained with the primary antiserum diluted in blocking solution for 12 h at 4 °C. After washing the stained samples with PBS-T three times for 15 min each, secondary antibodies were treated for 8 h at 4 °C. Then, samples were washed with PBS-T three times for 15 min each and mounted on slide glasses with mounting solution (50% glycerol in PBS-T). Detailed antibody information is as follows. Primary antibodies: mouse anti-nc82 (1:30, DSHB), mouse anti-GFP (1:500, Invitrogen), rabbit anti-GFP (1:500, Invitrogen), chicken anti-GFP (1:500, Invitrogen), and rabbit anti-RFP (1:500, Invitrogen). Secondary antibodies: goat anti-mouse Alexa 488 (1:250, Invitrogen), goat anti-mouse Alexa 568 (1:250, Invitrogen), goat anti-rabbit Alexa 488 (1:250, Invitrogen), goat anti-rabbit Alexa 568 (1:250, Invitrogen), and goat anti-chicken Alexa 405 (1:250, Invitrogen). All confocal microscope images were collected by using Zeiss LSM700 laser-scanning confocal microscopy. Images were processed and analyzed by using ImageJ.

4.4. Electrophysiology

Extracellular single-unit recordings were performed using the tip-recording method [50]. 3 to 6-day-old flies, which are the appropriate age for taste physiological recording experiments, were briefly ice-anesthetized and their forelegs were removed. We used both male and female flies. A reference electrode containing *Drosophila* Ringer solution was inserted into the fly through the thorax to the tip of the labellum. To obtain neuronal signals, a labellar taste sensillum was contacted to a recording electrode (10–20 μm diameter) filled with tastant dissolved in 30 mM tricholine citrate solution (Sigma-Aldrich). The recording electrode was connected to TastePROBE (Syntech) with silver wire, and the neuronal signals from taste sensillum were collected by an acquisition controller (Syntech). Signals were amplified (10×), filtered (100–3000 Hz), and sampled at 12 kHz. LabChart 8 software (ADInstrument) was used to analyze neuronal firing rates. The neuronal firing rates were calculated by counting and doubling the number of spikes obtained in the first 500 ms after contact.

4.5. Two-way preference assay

The binary choice assay was performed with minor modifications of the original protocol [51,52]. 1 to 3-day-old flies were collected in groups of 50 males and placed in a fresh vial for 1 day. Flies were starved for 20 h in a 1% agarose vial prior to the assay. Tastants were mixed into 1% agarose containing either blue dye (Brilliant Blue FCF, wako, 0.125 mg/ml) or red dye (sulforhodamine B, sigma, 0.5 mg/ml). 72-well plate dishes were filled with tastant in alternating color patterns. Starved flies were transferred to the 72-well plate dishes and allowed to feed for 90 min at room temperature in the dark. The fed flies were frozen at – 20 °C. The abdomen color of fed flies was observed under a dissection microscope. A preference index (PI) was calculated by the equation: $PI = (N_R + N_P/2)/(N_R + N_P + N_B)$ or $(N_B + N_P/2)/(N_R + N_P + N_B)$, where N_R is the number of red, N_P is the number of purple, and N_B is the number of blue abdomens. PI 0.5 means no preference for either food. No effects were observed for the dyes alone.

4.6. Data statistics and analysis

All statistical analyses were performed with GraphPad Prism 9 software. All error bars represent the standard error of the mean (SEM). Ordinary one-way ANOVA followed by Dunnett's multiple comparison test was used. a: $p < 0.05$, b: $p < 0.005$, c: $p < 0.001$. Statistical analysis compared the mean of each group with the mean of control w^{1118}.

Ethical statement

This manuscript does not involve any ethical issues.

Data availability statement

Data and fly stocks will be made available on request.

CRediT authorship contribution statement

Keehyun Park: Writing – original draft, Investigation. **Hyungjun Choi:** Writing – original draft, Investigation. **I Joon Han:**

Investigation. **Wayessa Rahel Asefa:** Investigation. **Chaiyoung Jeong:** Investigation. **Seungyun Yu:** Investigation. **Hanhee Jeong:** Investigation. **Minkook Choi:** Investigation. **Sung-Eun Yoon:** Investigation. **Young-Joon Kim:** Investigation. **Min Sung Choi:** Writing – original draft, Conceptualization. **Jae Young Kwon:** Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29358.

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