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Molecules and Cells

A Systematic Analysis of *Drosophila* Gustatory Receptor Gene Expression in Abdominal Neurons which Project to the Central Nervous System

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In *Drosophila*, the gustatory receptor (*Gr*) gene family contains 60 family members that encode 68 proteins through alternative splicing. Some gustatory receptors (Grs) are involved in the sensing of sugars, bitter substrates, CO_2, pheromones, and light. Here, we systematically examined the expression of all 68 Grs in abdominal neurons which project to the abdominal ganglion of the central nervous system using the *GAL4/UAS* system. *Gr* gene expression patterns have been successfully analyzed in previous studies by using the *GAL4/UAS* system to drive reporter gene expression. Interestingly, 21 *Gr-GAL4* drivers showed abdominal ganglion projection, and 18 of these 21 *Gr-GAL4* drivers labeled multidendritic neurons of the abdominal wall. 4 drivers also labeled neuronal processes innervating the reproductive organs. The peripheral expression of *Gr-GAL4* drivers in abdominal multidendritic neurons or neurons innervating the reproductive organs suggests that these Grs have atypical sensory functions in these organs not limited to conventional taste sensing.

INTRODUCTION

The chemosensory system in animals is used to detect changes in the environment (van der Goes van Naters and Carlson, 2006). *Drosophila melanogaster* provides a relatively easily manipulated genetic system to study chemosensation. Molecular studies of *Drosophila* chemosensation were initiated upon molecular identification of the odorant and gustatory receptors (Clyne et al., 1999; 2000; Vosshall et al., 1999).

68 *Drosophila* gustatory receptors are made from 60 gustatory receptor (*Gr*) genes, due to alternatively spliced forms (Clyne et al., 2000; Dunipace et al., 2001; Robertson et al., 2003; Scott et al., 2001). Many studies have focused on the expression and function of these *Gr*s in taste organs that have access to external sensory cues including the labellum, the pharyngeal organs, and the tarsi (Isono and Morita, 2010; Thorne et al., 2004; Wang et al., 2004; Weiss et al., 2011). *Gr5a*, *Gr64a*, and *Gr64f*, which are members of a *Gr* subfamily of eight *Gr*s, were found to act as sugar sensors through genetic approaches (Dahanukar et al., 2007; Jiao et al., 2008; Slone et al., 2007), and *Gr33a*, *Gr66a*, and *Gr93a* were found to be required for responses to caffeine and certain other bitter compounds (Lee et al., 2009; Moon et al., 2006; 2009).

Gr functions are not limited to conventional taste sensing. Genetic approaches disrupting the activity of *Gr68a*-expressing neurons, *Gr32a*, *Gr33a*, or *Gr39a*, cause alterations in courtship behavior, consistent with a role in pheromone detection (Bray and Amrein, 2003; Miyamoto and Amrein, 2008; Moon et al., 2009; Watanabe et al., 2011). *Gr21a* and *Gr63a* together mediate the CO_2 response in the antenna (Jones et al., 2007; Kwon et al., 2007). *Gr28b* was recently found to be critical for light-induced responses in the *Drosophila* larvae (Xiang et al., 2010), consistent with the light-sensing role of its *C. elegans* homolog *lite-1* (Liu et al., 2010). *Gr28a* and five alternatively spliced forms of the *Gr28b* gene were found to be expressed in various taste and non-taste tissues such as the abdominal multidendritic neurons, putative hygroreceptive neurons of the arista, neurons associated with the Johnston's organ, peripheral proprioceptive neurons in the legs, neurons in the larval and adult brain, and oenocytes (Thorne and Amrein, 2008). Thus, *Gr* genes appear to be utilized in non-gustatory sensory roles in addition to their gustatory roles, such as olfaction, light sensing, proprioception, hygroreception, and other sensory modalities in the nervous system and other tissues.

Here, to further examine potential non-gustatory sensory roles of the *Gr* genes in abdominal tissues, we systematically investigate the expression of all 68 members of the *Drosophila* Gr family in the abdomen, including abdominal ganglion projection, and expression in the abdominal wall and reproductive organs. 67 *Gr-GAL4* drivers representing the 68 Grs were used to examine expression. Our study provides insight into potential non-conventional roles of *Drosophila Gr* genes in the abdominal wall and reproductive organs.

MATERIALS AND METHODS

Drosophila stocks and transgenic flies

Flies were grown on standard cornmeal/agar culture medium at an average culture temperature of 23°C. All 67 *Gr-GAL4* trans-

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genic lines used in this study were previously described (Weiss et al., 2011). 67 drivers were used to assess the expression of the 68 Gr proteins; two alternative transcripts of *Gr23a* share the same promoter. *UAS-mCD8-GFP* was used as a GFP reporter to visualize expression of the *GAL4* transgenes (Lee and Luo, 1999). mCD8-GFP is a membrane marker which allows visualization of entire cell shapes.

Dissection, antibody staining, and imaging

2- or 3-day-old flies were dissected to examine reporter expression, and males and females were examined separately.

To examine expression in thoracic-abdominal ganglia, dissected thoracic-abdominal ganglia were subjected to antibody staining (Dahanukar et al., 2007). To examine expression in the reproductive organs, whole abdomens were first stained, and the abdomen was sliced at the ventral side to dissect reproductive organs out of the abdominal cavity while mounting, to facilitate visualization. To examine expression in the abdominal wall, stained whole abdomens were sliced at the ventral or lateral side and spread open while mounting. The internal organs were removed to visualize multidendritic neurons tiling the dorsal or lateral abdominal wall. Expression was not observed in the ventral abdominal wall. Multidendritic neuron expression was initially examined with ventrally sliced samples and later examined again with laterally sliced samples to verify the lack of expression in the ventral side while facilitating image acquisition of lateral multidendritic neurons.

Antibody staining was adapted from Laissue et al. (1999). The primary antibodies used are as follows: rabbit anti-GFP (1:1000) (Molecular Probes); nc82 monoclonal antibody (1:100) (a gift of Dr. Alois Hofbauer, University of Regensburg). The secondary antibodies used were goat anti-mouse and goat anti-rabbit IgG conjugated to either Alexa 568 or Alexa 488 (1:1000) (Molecular Probes).

All images were collected on a Zeiss LSM 510 laser-scanning confocal microscope.

RESULTS

Expression of all 68 Grs was systematically examined in the abdomen using the *GAL4/UAS* system

We systematically examined expression of all *Drosophila* Grs in the abdomen, using 67 *Gr-GAL4* transgenes which represent the 68 gustatory receptors to drive expression of a GFP reporter; two alternative transcripts of *Gr23a* share the same promoter. *In situ* hybridization with *Gr* genes has been mostly unsuccessful (Clyne et al., 2000; Dahanukar et al., 2007; Dunipace et al., 2001; Moon et al., 2009; Scott et al., 2001), likely due to low expression levels, and thus the *GAL4/UAS* system has been more widely utilized to analyze *Gr* expression patterns (Brand and Perrimon, 1993; Chyb et al., 2003; Dunipace et al., 2001; Moon et al., 2009; Scott et al., 2001; Thorne and Amrein, 2008). *Gr-GAL4* expression patterns in the adult labellum correspond well with functional analysis (Weiss et al., 2011), validating this approach. Since expression driven by *Gr-GAL4* transgenes can be variable depending on the independent insertion line, we initially selected and used representative lines for each *Gr-GAL4* driver that were previously observed to be the most consistent in expression levels and pattern, with high penetrance (A.D., J.Y.K., L.W., F. L., J-H.P., and J.R.C., unpublished results) (Weiss et al., 2011). The 67 *Gr-GAL4* drivers were systematically examined for expression in the abdominal ganglion, the abdominal wall, and the internal male and female reproductive organs. When expression was observed in the abdomen, this expression was verified in at least

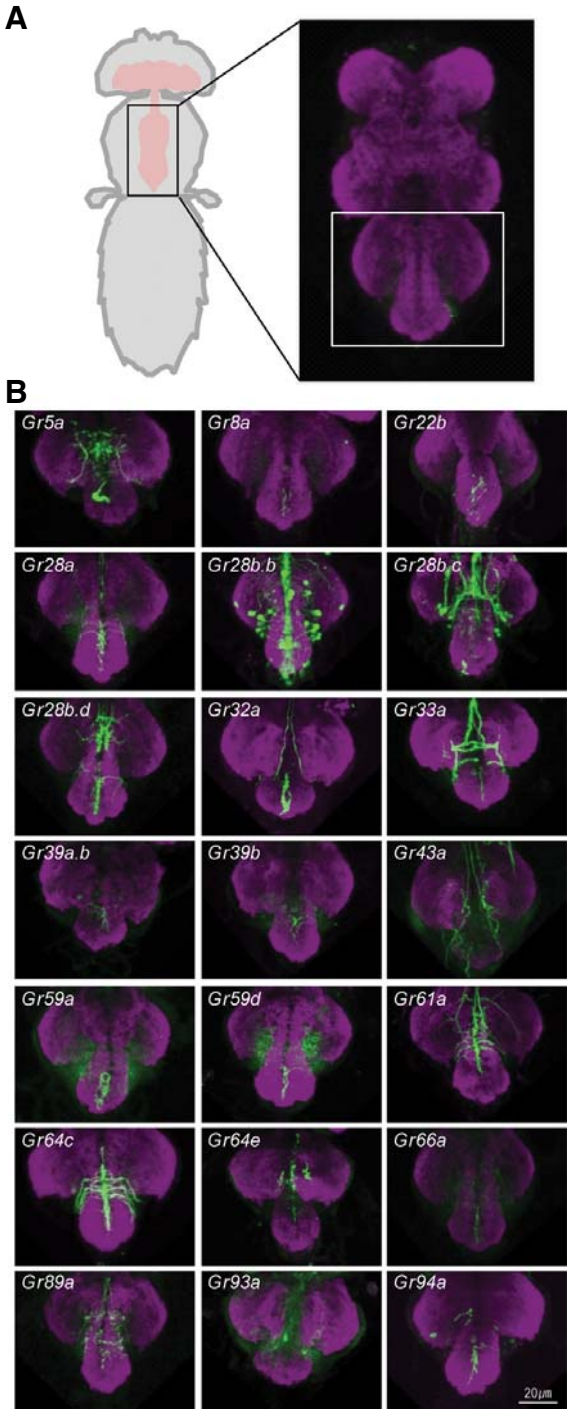

Fig. 1. 21 *Gr-GAL4* drivers are expressed in abdominal ganglion projections. (A) Schematic and confocal image of an adult thoracic-abdominal ganglion stained with the monoclonal antibody nc82 to label the neuropil (magenta). The abdominal ganglion is the neuromere located at the posterior-most end of the thoracic-abdominal ganglion. The thoracic T3 neuromere and abdominal ganglion regions (indicated by the white box in the confocal image) are shown in (B). (B) Confocal images of 21 *Gr-GAL4* drivers which drive GFP reporter expression in projections to the abdominal ganglion. GFP signals were detected by anti-GFP antibody (green) and neuropil detected using nc82 antibody (magenta). Anterior is up and posterior is down for this figure and all subsequent figures.

one additional independent transgenic line. Additional lines were not available for the *Gr28b.d*-, *Gr64c*-, and *Gr66a-GAL4* drivers.

Transgenic flies containing 2 copies of each *Gr-GAL4* transgene and 2 copies of *UAS-mCD8-GFP* were examined for expression, with the exception of *Gr36c-GAL4* whose transgene insertion homozygotes are lethal. As negative controls, w^{1118} and w^{1118}*; UAS-mCD8-GFP; UAS-mCD8-GFP* (a total of four copies of the *UAS* transgene) flies were stained as per the described protocol. In w^{1118}, the background used to generate the *Gr-GAL4* transgenic lines (Weiss et al., 2011), no GFP expression was observed. In the w^{1118}*; UAS-mCD8-GFP; UAS-mCD8-GFP* flies, background levels of GFP much lower than what we observed with the *Gr-GAL4* drivers in this study were seen, with the exception of the male testes which show relatively strong non-specific GFP expression (Fig. 3A). This non-specific GFP expression precluded observation of *Gr-GAL4* driver expression in cells of the male testes.

21 *Gr-GAL4* drivers show abdominal ganglion projection

The brain and thoracic-abdominal ganglia compose the *Drosophila* central nervous system, and the abdominal ganglion (AG) is a likely CNS target of neurons innervating abdominal organs (Nassel, 1996; Stocker, 1994). When the 67 drivers were examined, 21 were observed to have GFP-positive neuronal projections to the AG (Fig. 1B): *Gr5a, Gr8a, Gr22b, Gr28a, Gr28b.b, Gr28b.c, Gr28b.d, Gr32a, Gr33a, Gr39a.b, Gr39b, Gr43a, Gr59a, Gr59d, Gr61a, Gr64c, Gr64e, Gr66a, Gr89a, Gr93a,* and *Gr94a*. The remaining 46 drivers either did not show projection to the thoracic-abdominal ganglia, or showed projection to only the thoracic ganglia from tarsal sensory neurons (A.D., J.Y.K., L.W., F. L., J-H.P., and J.R.C., unpublished results). Sexual dimorphism was not observed for any of the 67 *Gr-GAL* drivers (data not shown).

Each of the 21 *Gr-GAL4* drivers that show AG neuronal projection has a characteristic projection pattern and differing signal intensities (Fig. 1B). Due to technical limitations in performing double labeling, we were not able to determine AG co-localization among the *Gr-GAL4* drivers for further detailed subclassification.

Gr-GAL4 drivers are expressed in multidendritic neurons of the abdominal wall

Gr-GAL4 drivers were also observed to label multidendritic neurons innervating the abdominal wall (Table 1, Fig. 2). Multidendritic (md) neurons are neurons with multiple dendrites that lie beneath the body wall, which are divided into three subtypes in the embryo and larva based on dendrite morphology and the targets they innervate: dendritic arborization (md-da) neurons are the most abundant subtype with extensive dendritic arborizations, bipolar dendrite (md-bd) neurons have bipolar dendrites growing in opposite directions, and tracheal dendrite (md-td) neurons have several dendrites that innervate the trachea (Bodmer and Jan, 1987). The md-da neurons have been classified further into four subtypes in the larva based on peripheral dendritic morphology, with class I da neurons having the simplest morphology, and class IV da neurons with the most complex dendritic arbors (Grueber et al., 2002). During metamorphosis, the larval da neurons undergo cell death or extensive remodeling of their arbors, and some adult-specific da neurons appear (Shimono et al., 2009).

Of the 67 drivers examined, 18 *Gr-GAL4* drivers were observed to drive expression in multidendritic neurons in the adult (Fig. 2). We classified the observed expression patterns into largely three patterns. Expression pattern A is the simplest

Table 1. *Gr-GAL4* expression in the abdominal ganglion and neurons in the abdominal wall

Gr gene	Lines	Projection to AG	Abdominal wall
Gr2a	1	-	-
Gr5a	2	++	C
Gr8a	2	++	-
Gr9a	1	-	-
Gr10a	1	-	-
Gr10b	1	-	-
Gr21a	1[a]	-	-
Gr22a	1	-	-
Gr22b	2	++	A
Gr22c	1[a]	-	-
Gr22d	1	-	-
Gr22e	1	-	-
Gr22f	1	-	-
Gr23a	1	-	-
Gr28a	3[b]	++	B
Gr28b.a	2	-	-
Gr28b.b	2	++	B
Gr28b.c	3	++	B/C
Gr28b.d	1[b]	++	B
Gr28b.e	1[a]	-	-
Gr32a	2	++	A
Gr33a	2	++	A
Gr36a	1	-	-
Gr36b	1	-	-
Gr36c	2	-	-
Gr39a.a	2	-	-
Gr39a.b	3	+	C
Gr39a.c	1	-	-
Gr39a.d	1	-	-
Gr39b	2	++	A
Gr43a	2	+	-
Gr47a	1[a]	-	-
Gr47b	1	-	-
Gr57a	1	-	-
Gr58a	1	-	-
Gr58b	1	-	-
Gr58c	2	-	-
Gr59a	3	+	A
Gr59b	1[b]	-	-
Gr59c	1	-	-
Gr59d	2	++	A
Gr59e	1	-	-
Gr59f	1	-	-
Gr61a	2	++	B
Gr63a	1	-	-
Gr64a	2	-	-
Gr64b	1	-	-
Gr64c	1	++	B
Gr64d(e)	1	-	-
Gr64e	2	++	B
Gr64f	1	-	-
Gr66a	1	++	C
Gr68a	1[b]	-	-
Gr77a	1	-	-
Gr85a	1	-	-
Gr89a	3	++	C
Gr92a	1	-	-
Gr93a	2	+	A
Gr93b	1	-	-
Gr93c	1	-	-
Gr93d	1	-	-
Gr94a	2	++	-
Gr97a	2	-	-
Gr98a	1	-	-
Gr98b	1	-	-
Gr98c	1	-	-
Gr98d	1	-	-

++ indicates consistent AG expression in all animals and all lines examined, and + indicates lower penetrance of GFP expression or expression observed in only one line.

[a]One line received from K. Scott

[b]One line received from H. Amrein

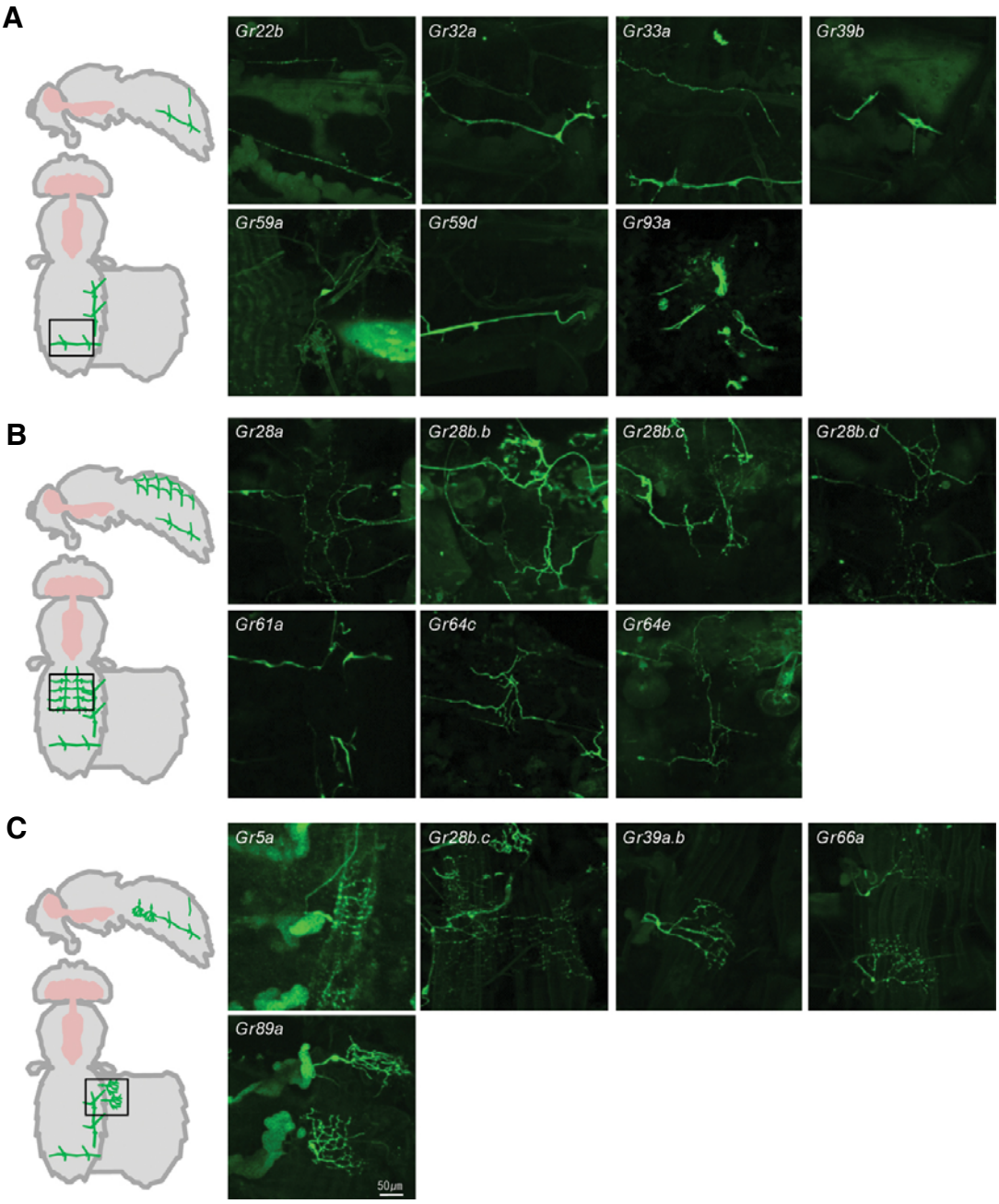

Fig. 2. 18 *Gr-GAL4* drivers express in multidendritic neurons of the abdominal wall. Expression patterns of the *Gr-GAL4* drivers observed to drive expression in multidendritic (md) neurons were classified into largely three patterns. Confocal images were taken using dissected flies with abdomens laterally sliced and spread open, with internal organs removed to facilitate imaging of the body wall. The regions shown in the confocal images are indicated by black boxes on the schematics. Samples were visualized using anti-GFP antibody. All lateral neuronal expression was observed symmetrically in both the left and right sides of dissected flies, but only one side is depicted in the schematics for simplicity. (A) Pattern A shows expression in md neurons with dorsal processes extending along the left-right axis, as well as in md neurons with lateral processes extending anteroposteriorly. These major processes have several protruding dendrites. (B) Pattern B shows expression in md neurons with ladder-like processes along the dorsal abdominal wall, in addition to pattern A neurons. (C) Pattern C shows expression in laterally localized md neurons with extensive arborization, in addition to pattern A neurons. Note that the *Gr28b.c-GAL4* driver shows both pattern B and pattern C expression.

pattern and is also a common feature of patterns B and C. *Gr-GAL4* drivers showing expression pattern A are expressed in multidendritic (md) neurons with dorsal processes extending along the left-right axis, as well as in md neurons with lateral processes extending anteroposteriorly (Fig. 2A). Both the left-right and anteroposteriorly extending processes have several protruding dendrites. *Gr-GAL4* drivers which show expression pattern B are expressed in md neurons with ladder-like proc-

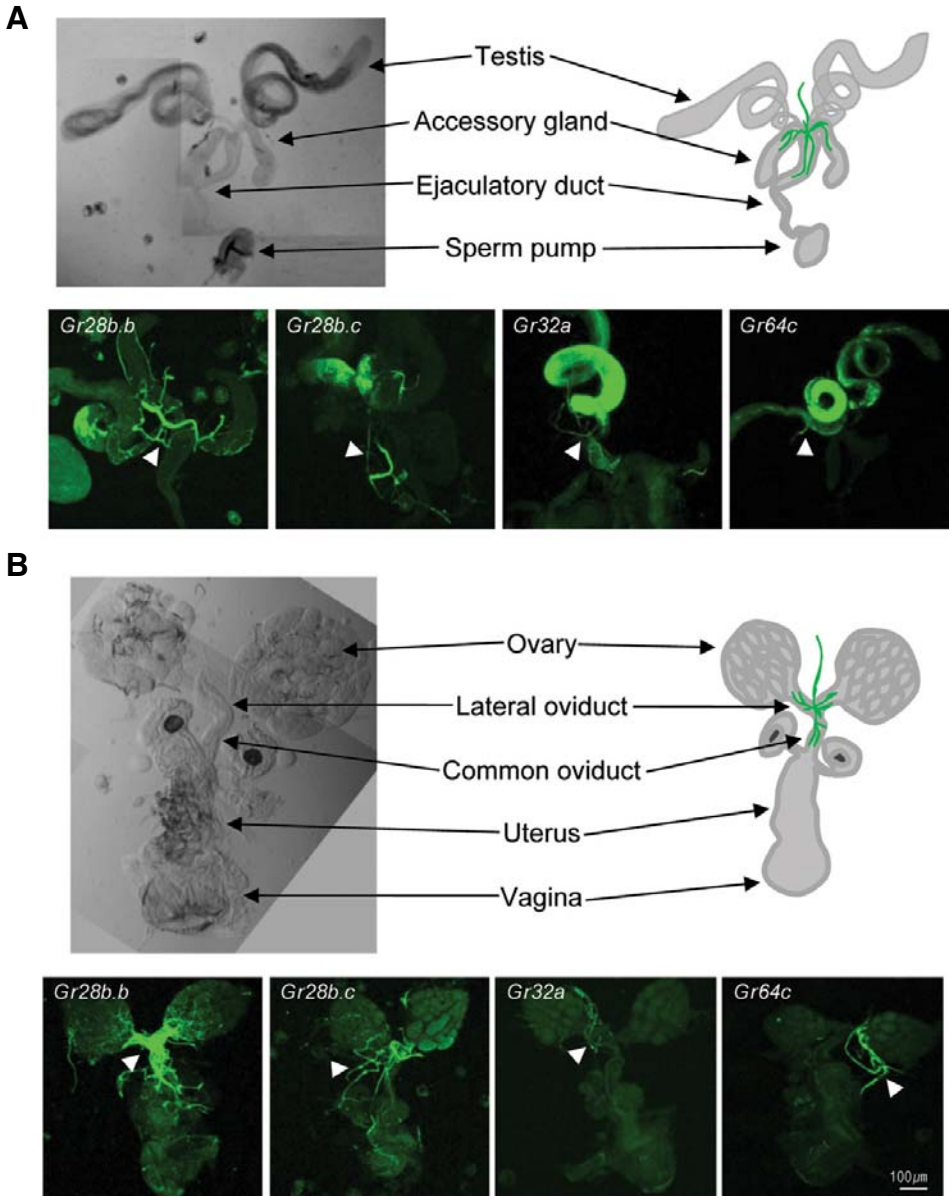

Fig. 3. 4 *Gr-GAL4* transgenes drive expression in neurons that appear to innervate the reproductive organs. Samples were visualized using anti-GFP antibody. (A) Confocal images of *Gr-GAL4* transgenes driving expression in neurons innervating male reproductive organs. Most neurons appear to innervate the accessory glands. (B) Confocal images of *Gr-GAL4* transgenes driving expression in neurons innervating female reproductive organs. Most neurons appear to innervate the common oviduct, lateral oviducts, or ovaries. Arrowheads indicate neurons expressing GFP.

esses along the dorsal abdominal wall in addition to pattern A neurons (Fig. 2B). *Gr-GAL4* drivers which show expression pattern C are expressed in laterally localized md neurons with extensive arborization in addition to pattern A neurons (Fig. 2C). All lateral neuronal expression was observed symmetrically in both the left and right sides of dissected flies.

All 18 *Gr-GAL4* drivers with expression in multidendritic neurons of the abdominal wall also showed expression in projections to the abdominal ganglion (Table 1), suggesting that the GFP-positive multidendritic neurons might project directly to the AG. Supporting this suggestion, we observed the direct projection of abdominal wall neurons expressing *Gr64c-GAL4* to the abdominal ganglion (Supplementary Fig. S1A).

Gr-GAL4 drivers appear to be expressed in neurons innervating internal reproductive organs

The *Drosophila* male reproductive organs include the testes, accessory glands, ejaculatory duct, and sperm pump (Fig. 3A), and female reproductive organs include the ovaries, lateral oviducts, common oviduct, uterus, and vagina (Fig. 3B).

4 *Gr-GAL4* drivers, *Gr28b.b*, *Gr28b.c*, *Gr32a*, and *Gr64c*, are expressed in neurons that appear to innervate both the male and female reproductive organs (Fig. 3). Neurons innervating the male reproductive organs appear to mainly innervate the accessory glands (Fig. 3A). The neurons innervating the female reproductive organs appear to mainly innervate the common oviduct, lateral oviducts, or ovaries (Fig. 3B). Expression in cells

of the reproductive organs themselves was not observed for all 67 drivers, although strong non-specific expression precluded detection in the male testes, as described above. Expression of these *Gr-GAL4* drivers was consistent in at least two independent *Gr-GAL4* lines, with the exception of *Gr32a-GAL4* for which reproductive organ-innervating expression was only observed in one independent line.

Gr28b.b-, *Gr28b.c*-, *Gr32a*-, and *Gr64c-GAL4* drivers also showed expression in neuronal projections to the AG (Fig. 1B). Of these, *Gr28b.b-GAL4*-expressing reproductive organ-innervating neurons were observed to directly project to the AG (Supplementary Fig. S1B). This supports the possibility that *Gr*-expressing neurons innervating the reproductive organs directly project to the abdominal ganglion.

DISCUSSION

Here, we systematically examined the expression of all Gr family members in the abdomen, focusing on expression in the abdominal ganglion, abdominal multidendritic neurons, and reproductive organs. Our analysis provides insight into the potentially diverse roles of the *Gr*s in the abdomen.

Gr-GAL4 driver-expressing multidendritic neurons and reproductive organ-innervating neurons likely project directly to the abdominal ganglion

Several lines of evidence suggest that the peripheral *Gr-GAL4* driver-expressing multidendritic neurons and neurons innervating the reproductive organs directly project to the abdominal ganglion. First, peripheral expression of *Gr-GAL4* drivers in multidendritic neurons tiling the abdominal wall, or in neurons innervating the reproductive organs appears to correlate well with expression in neurons projecting to the abdominal ganglion. 18 *Gr-GAL4* drivers were observed to drive expression in md neurons and/or neurons innervating reproductive organs (Figs. 2 and 3), and all of these drivers drove expression in neurons projecting to the abdominal ganglion (Table 1). Of the 21 drivers observed to drive expression in neurons projecting to the abdominal ganglion (Fig. 1), *Gr8a-, Gr43a*-, and *Gr94a-GAL4* drivers show AG projection but peripheral expression in multidendritic neurons or neurons innervating reproductive organs was not observed. These *Gr-GAL4* drivers may be expressed in other abdominal tissues that we did not focus on, or peripheral expression may be too weak for observation. Second, when more than two independent lines were examined for a certain *Gr-GAL4* transgene and expression was observed in only one independent line (Table 1), the line that showed AG projection invariably also showed peripheral expression, while the line(s) that did not show AG projections also did not show peripheral expression. Third, we obtained direct evidence that peripheral neurons expressing the *Gr-GAL4* transgenes project to the AG. As mentioned above, *Gr64c-GAL4* driver-expressing multidendritic neurons were observed to extend their processes directly to the AG, and *Gr28b.b-GAL4* driver-expressing neurons were observed to extend processes from the female reproductive organs directly to the AG (Supplementary Fig. S1). Direct projections are not easy to observe, due to difficulties in preparing dissected samples with AG, abdominal wall or reproductive organs, and neural fibers all intact.

The abdominal ganglion is an organ in insects that regulates functions such as respiration, heartbeat, hindgut movement, abdominal posture, and functions of the genitalia and ovipositor (Nassel, 1996). In studies on *Drosophila* ovulation, female postmating behavior, and male courtship or reproductive behavior (Hasemeyer et al., 2009; Lee et al., 2001; Monastirioti, 2003; Yang et al., 2009), abdominal peripheral neurons were observed to directly project to the abdominal ganglion to mediate diverse functions. Therefore, although it is not yet clear what functions the *Gr*s are mediating in the multidendritic neurons and reproductive organs, it seems likely that the information sensed by the *Gr*s in these organs is conveyed to the abdominal ganglion to be processed.

Potential atypical functions of Grs in the abdominal wall and reproduction

18 *Gr-GAL4* drivers were observed to be expressed in multidendritic neurons of the abdominal wall in characteristic patterns. The expression of *Gr28a*-, *Gr28b.a*-, *Gr28b.b*-, *Gr28b.c*-, *Gr28b.d*-, and *Gr66a-GAL4* drivers in multidendritic abdominal neurons is consistent with previous observations (Shimono et al., 2009; Thorne and Amrein, 2008). Functions of the multidendritic neurons have mainly been explored at the larval stage, but are virtually unknown at the adult stage. Bipolar dendritic (md-bd) neurons and class I md-da neurons were found to be required for the propagation of rhythmic peristaltic muscle movement needed for proper larval locomotion, and were proposed to be proprioceptors (Hughes and Thomas, 2007; Song et al., 2007). Class IV neurons have also been implicated in larval locomotion (Ainsley et al., 2003). Class IV md-da neurons were shown to function in thermal and mechanical nociception as well as light sensing in larva (Hwang et al., 2007; Xiang et al., 2010). *Gr28b* is required for proper light transduction in class IV neurons in larva, although it is unclear if any of the alternatively spliced forms of *Gr28b* act as the actual light receptor (Xiang et al., 2010). Much is unknown about multidendritic neuron biology in the adult, including whether larval functions are preserved at the adult stage. Our study provides clues that can facilitate functional studies to shed light on the roles of individual *Gr*s or groups of *Gr*s in the adult multidendritic neurons.

4 *Gr-GAL4* drivers, *Gr28b.b*, *Gr28b.c*, *Gr32a*, and *Gr64c*, are expressed in neurons that appear to innervate both the male and female reproductive organs (Fig. 3). Based on expression pattern, it seems likely that the neurons expressing these *Gr*s could function in regulation of accessory gland secretion in males or regulation of ovulation in females. Also, female postmating behavior is regulated upon detection of male sex peptide by receptors in sensory neurons innervating the female genital tract (Hasemeyer et al., 2009; Yang et al., 2009), suggesting that *Gr*-expressing neurons innervating the reproductive organs may have similar functions. Although sex-specific expression was not observed for any of the *Gr-GAL4* drivers, it seems plausible that second order neurons may confer specificity in relaying information to higher centers, similar to what has been proposed for *Gr32a* (Koganezawa et al., 2010).

Among the *Gr*s observed to express in neurons apparently innervating the reproductive organs, *Gr32a* is required for pheromone detection (Miyamoto and Amrein, 2008; Wang et al., 2011), and *Gr64c* is a member of the putative sugar receptor subfamily (Dahanukar et al., 2007). It is as yet unclear whether these *Gr*s act as chemosensors for certain molecules in the reproductive organs, or if they act as sensors for completely different modalities.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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