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Engrailed expression in subsets of adult *Drosophila* sensory neurons:

an enhancer-trap study

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Abstract

Engrailed (En) has an important role in neuronal development in vertebrates and invertebrates. In adult *Drosophila*, although En expression persists throughout adulthood, a detailed description of its expression in sensory neurons has not been made. In this study, *en-GAL4* was used to drive *UAS-CD8::GFP* expression and the projections of sensory neurons were examined with confocal microscopy. En protein expression was confirmed using immunocytochemistry. In the antenna, En is present in subsets of Johnston's organ neurons and of olfactory neurons. *En*-driven GFP is expressed in axons projecting to 18 identified olfactory glomeruli, originating from basiconic, trichoid and coeloconic sensilla. In most cases both neurons of a sensillum express En. En expression overlaps with that of Acj6, another transcription factor. *En*-driven GFP is also expressed in a subset of maxillary palp olfactory neurons and in all mechanosensory and gustatory sensilla in the posterior compartment of the labial palps. In the legs and halteres, *en*-driven GFP is expressed in only a subset of the sensory neurons of different modalities that arise in the posterior compartment. Finally, *en*-driven GFP is expressed in a single multidendritic sensory neuron in each abdominal segment.

Keywords

Transcription factor; homeodomain; *Drosophila melanogaster*; sense organs

Introduction

Engrailed (En) is a homeodomain-containing transcriptional regulator that has an important role in nervous system development. It is found ubiquitously in bilaterian animals, indicating that it arose more than 500 million years ago, in the Cambrian era (Gibert, 2002, Webster and Mansour, 1992). En was first identified in *Drosophila*, where it was shown to play a crucial part in the early patterning of body segments and limbs (Kornberg, 1981, Lawrence and Struhl, 1982, Morata and Lawrence, 1975); it was subsequently found also to have a role in vertebrate limb development (Wurst et al., 1994).

However, the most conserved role of En appears to be in neuronal development. En has a role in cerebellar patterning (Baader et al., 1999), and the gradient of En in the tectum activates ephrin expression and guides retinal axons (Friedman and O'Leary, 1996, Itasaki and Nakamura, 1996, Logan et al., 1996, Shigetani et al., 1997). En regulates axonal pathfinding in spinal cord interneurons (Saueressig et al., 1999) and may influence their synaptic connections to motor neurons (Wenner et al., 2000). In addition, it affects the survival of dopaminergic midbrain neurons through its effects on α -synuclein (Simon et al., 2001) and has recently been linked to autism spectrum disorder (Kuemerle et al., 2007).

In the embryonic *Drosophila* nervous system, En controls neuron/glia fate decisions, establishment of neuronal identity and axon pathfinding (Bhat and Schedl, 1997, Condrón et al., 1994, Joly et al., 2007, Lundell et al., 1996), the latter in part via its repression of the cell-surface recognition molecules Neuroglian and Connectin (Siegler and Jia, 1999), and by activation of the netrin receptor, Frazzled (Joly et al., 2007). Recently, we showed that En controls the shape of axonal arborizations and, more importantly, synaptic choice in a circuit of identified neurons; the cercal afferent to giant interneuron synapses of the cockroach, *Periplaneta americana* (Marie et al., 2000, Marie and Blagburn, 2003, Marie et al., 2002).

However, although a lot is known about the role of En in establishing compartmental and neuronal identity during embryonic and pupal development, surprisingly little is known about its possible roles later on. At a gross anatomical level, it is known that En expression in body segments and appendages of *Drosophila* persists into adulthood (Hama et al., 1990, Rogina and Helfand, 1997) and it has been observed in some sensory and central neurons of adult cockroaches and grasshoppers (Blagburn et al., 1995, Siegler et al., 2001). However, the extent and functions of this adult neuronal En expression are not known. The genetic tools available for *Drosophila* make it the best system to investigate this question. The focus of this study is to describe the En-expressing neurons in the adult, concentrating in particular on the sensory neurons and their axonal projections.

Methods

En-driven GFP flies

Fruit flies (*Drosophila melanogaster*) were maintained in standard corn meal-agar medium or yeasted 4-24 Instant Medium (Carolina Biological Supply), in plastic vials at room temperature (22°C). The following stocks were obtained from the Bloomington Stock Center: *y¹ w^{*}*; *P{en2.4-GAL4}e16E* (Yoffe et al., 1995) and *y¹ w^{*}*; *P{UAS-mCD8::GFP.L}LL5* (Lee and Luo, 1999). These were crossed and live progeny were initially checked for GFP epifluorescence using a Zeiss microscope equipped with Lucifer yellow filters.

DiI retrograde labeling

Haltere sensilla were retrogradely labeled by applying a droplet of DiI (1,1'-dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine perchlorate: Molecular Probes) dissolved in dimethyl formamide to the interior of the thoracico-abdominal ganglion of a formaldehyde-fixed semi-intact preparation, using a broken-tipped glass micropipette. The preparation was left for 48-72 h in fixative at room temperature, then mounted in Vectastain and rapidly examined with the confocal microscope before the dye diffused.

Immunohistochemistry

Adults were used soon after eclosion (approx. 4h) and at later times (up to 1 week). The animals were anesthetized by cooling, then immersed in fixative (4% paraformaldehyde in 0.1M PBS buffer). For antibody staining of antennae, the heads of pupae at stages P5-P7 (Bainbridge and Bownes, 1981) were removed and placed in fixative. Alternatively, fixed adult heads were embedded in Tissue-Tek OCT medium, frozen, and 20 µm frontal sections cut using a cryotome. All tissues were fixed for 30-60 min, then washed in buffer for approximately 1-2 h. Tissues were first incubated in normal horse serum in PBS + 0.3% Triton X 100 (PBST) for 1 h, then in primary antibody, diluted in PBST, for 48 h at 4°C. 4D9 antibody was obtained from Dr. Corey Goodman or from the Developmental Studies Hybridoma Bank (DSHB) and used at a dilution of 1/10. Anti-Acj6 and nc82 (anti-Bruchpilot) antibodies were obtained from the DSHB and also used at 1/10. After 4 × 15 min washes, Cy5-labeled horse anti-mouse antibody was applied at a dilution of 1/200 for 16-20 h at 4°C, and the tissue was again washed

4 times. The specimens were washed in PBS then cleared and mounted in Vectashield, then examined with a Zeiss Pascal laser scanning confocal microscope.

Image stacks were imported into ImageJ (Wayne Rasband, NIH), where they were adjusted for optimal contrast. After color channel separation, in some regions portions of the stack were masked using the paintbrush tool, in order to remove overlying background fluorescence, or to create false color masks. Maximum intensity z-series projections of recombined color stacks were imported into Adobe Photoshop for construction of figures. Display colors were adjusted for optimal color-deficit presentation using the Photoshop Vischeck plugin (Vischeck.com).

Results

Adult flies of genotype *en-GAL4 / UAS-mCD8::GFP* were examined using laser scanning confocal microscopy. GFP fluorescence appeared in a similar pattern to that described for β -galactosidase activity in *ryxho25* flies (Hama et al., 1990), with strong expression in antennae, the maxillary and labial palps of the proboscis, ocelli, the legs, wing hinge, haltere and abdominal stripes (Fig. 1). In addition, GFP was seen in a single direct flight muscle (probably the axillary levator), the hindgut, and in the genitalia. Fluorescence was strongest shortly after eclosion (approx. 4h) but persisted in the same pattern for at least 1 week, particularly in sensory neurons.

GFP driven by *engrailed* in the antenna and proboscis

One of the most notable sensory organs in which *en*-driven GFP was present is the antenna, in the three distal segments; the pedicel, funiculus and arista (Fig. 2A). In the pedicel, a large cluster of Johnston's organ mechanoreceptor neurons expressed *en*-driven GFP (Fig. 2A, B, C). To confirm that GFP expression was truly representative of *en* gene activity, late pupal antennae were stained with the 4D9 antibody, which recognizes both the Engrailed and Invested (*Inv*) proteins (Patel et al., 1989a). *Inv* is a duplicate, or paralogue, of *En*, is largely co-expressed with it, and appears to have similar functions (Hanks et al., 1995), although there is also evidence for small differences in CNS expression patterns (Siegler and Jia, 1999). It now appears that all insects have two *En/Inv* paralogues (Peel et al., 2006). In the case of the cockroach, the paralogues have been shown to have subtly different effects on sensory axon guidance and synaptic connectivity (Marie and Blagburn, 2003).

All Johnston's organ receptors that expressed GFP were also immunopositive for *En/Inv* proteins (Fig. 2B), although the intensity of staining was not necessarily proportional to the GFP fluorescence intensity. Some cells in the wall of the pedicel were *En/Inv*-immunopositive but did not contain GFP; these probably represent cells that express only the *invested* gene. Optically reslicing the pedicel showed that GFP-expressing Johnston's organ neurons were almost completely confined to the posterior receptor group (Gopfert and Robert, 2002; Kamikouchi et al., 2006) (Fig 2C).

In the base of the arista, a cluster of 5-10 large GFP-expressing neurons extend dendrites midway along the length of the arista (Fig. 2A, B). These neurons were also strongly *En/Inv*-immunopositive (Fig. 2B) and are probably hygroreceptors (Sayeed and Benzer, 1996).

In the funiculus, many cells contained *en*-driven GFP fluorescence, and all of these were, at least to some degree, *En/Inv*-immunopositive (Fig. 2B). The sensory neurons in the funiculus are known to be olfactory neurons (Stocker, 1994). In all olfactory sensilla examined in detail there was more than one GFP-expressing cell, at least one of which gave rise to an axon. Innervating what appeared to be trichoid sensilla were two GFP-expressing cells, one intensely stained with 4D9 and the other fainter (Fig. 2D). Underlying smaller basiconic sensilla were three GFP-expressing cells, again with one containing more *En/Inv* immunoreactivity than the

others (Fig. 2E). Two dendrites could be observed projecting into the basiconic hair. In addition, many *en*-driven GFP-expressing cells were clustered around all three chambers of the sacculus (Shanbhag et al., 1995).

One of the putative binding targets of Engrailed is the gene *altered chemosensory jump 6* (*acj6*) (Solano et al., 2003), which encodes for a transcription factor that is also expressed in the olfactory neurons (Komiyama et al., 2004). Staining of frozen sections of funiculi with antibody against Acj6 protein showed many immunopositive olfactory neuronal nuclei (Fig. 2F). A subset of these cells expressed *en*-driven mCD8::GFP.

In the proboscis, mCD8::GFP driven by *en-GAL4* is expressed in a population of cells innervating basiconic sensilla in the maxillary palps (Fig. 2G). No expression was seen in the large mechanosensory sensilla. GFP expression was also observed in gustatory neurons underlying taste bristles in the posterior region of the labial palps (Fig. 2H, I). There are three types of taste bristle on the outer surface of the labial palp: large, small (curved), and the intermediate type; *en*-driven GFP expression was observed in all the neurons of all three sensillum types in the posterior third of the palp (Fig. 2I). Referring to the map of labellar sensilla (Hiroi et al., 2002, Hiroi et al., 2004), GFP-labeled sensilla are l6-l9, i9 and i10, s8-s12, and two, singly-innervated, mechanosensory bristles. The taste pegs on the inner surface do not appear to have GFP expression.

Sensory projections in the brain

mCD8::GFP expression driven by *en-GAL4* was visible in the central regions of the adult brain, with no labeling in optic lobes. 4D9 antibody staining confirmed that GFP-expressing neurons also contained some nuclear En/Inv protein; a few neurons in the subesophageal ganglion presumably contained Inv only since they did not express GFP (Fig. 3A). Two bilateral clusters of cell bodies were present in the protocerebrum, and other prominent bilateral and midline clusters in the subesophageal ganglion (Fig. 3A). The latter gave rise to prominent large interneurons that arborized in the subesophageal ganglion (Fig. 3B), and that appeared to send prominent axons down the cervical connective to the thoracicoabdominal ganglia (see Fig. 6D). There was also prominent GFP expression in axons projecting to the olfactory lobe from antennal olfactory neurons and maxillary palp olfactory neurons. GFP was also observed in mechanosensory neuropil in axons from Johnston's organ auditory neurons, and in subesophageal proboscis gustatory projections (Fig. 3B).

en-driven GFP-labeled olfactory projections

GFP-labeled olfactory afferents were observed projecting to several glomeruli of the olfactory lobe. To characterize the pattern of projections in more detail, brains were counterstained with the nc82 antibody that labels the presynaptic protein Bruchpilot (Wagh et al., 2006) - this enables the stereotyped pattern of glomeruli to be seen (Couto et al., 2005, Laissue et al., 1999, Vosshall and Stocker, 2007). Serial confocal sections through the olfactory lobes (Fig. 4A) revealed a stereotyped pattern of *en*-driven GFP fluorescence that was consistent in all the animals examined (N = 5 male + 6 female brains). From anterior, GFP-labeled axons were seen to project into the glomeruli DA2, VA6, VA7l, and VA5, with fainter labeling in VA7m and DA4m (Figs. 4A and 4B). Behind those glomeruli were axons in DC2, DC1, VM2, VM3 (Fig. 4C), followed by VC1, the faintly stained DM3, and the very brightly fluorescent DL5 (Figs. 4D). At the posterior of the olfactory lobe were the labeled glomeruli DC4, VM1, VM6 and the fainter DP1m and VL2p (Fig. 4E). It should be noted that the small fluorescent cell bodies lateral to the olfactory glomeruli in Figs. 4E-H in fact project to the region of neuropil dorsal to the olfactory lobes, not to the glomeruli themselves.

Comparison of the olfactory glomeruli to which *en*-driven GFP-expressing neurons project, coupled with the morphological classification of sensillum types on the funiculus, and data from previous studies (Couto et al., 2005, Vosshall and Stocker, 2007), allows the *En*-expressing olfactory sensilla to be identified (Table 1). GFP is seen underlying basiconic sensilla, and GFP-expressing afferents project to glomeruli that are innervated by antennal basiconic sensilla of types 4, 5, 6, and 8 (Table 1). However, none of these sensilla have more than two neurons (Couto et al., 2005), so one of the three GFP-expressing cells must be a glial or support cell. Similarly, GFP was observed in trichoid sensilla on the antenna, and GFP-expressing afferents project to glomeruli that are innervated by two of three neurons in the at3 type of sensillum. In this case, therefore, one neuron (the one that projects to DA4l, presumably) does not express *en*-driven GFP (see Fig. 2D). In addition, GFP-labeled afferents projected to glomeruli that are innervated by some of the coeloconic and the intermediate type of sensilla, but in these the receptor molecule complement is not yet known. Finally, GFP-expressing basiconic sensilla were prominent on the maxillary palp; the neurons of the pb2 type of basiconic sensillum project to glomeruli that are both GFP-labeled. Thus, we can conclude that *En* expression does not distinguish between sensilla of different types or sizes in any clear-cut way. All thin basiconic sensilla express *En* (apart from the second neuron of ab6), but then so does one type of small basiconic sensillum (ab8). Finally, in 5 out of 7 cases *En* is expressed in both the neurons of a sensillum.

GFP driven by *engrailed* in the leg sensory neurons

GFP expression was examined in the legs using confocal microscopy. Detailed descriptive efforts were concentrated on the prothoracic legs; however, the other legs had a very similar distribution of sensory neurons. A few hours after eclosion, the legs exhibit *en*-driven GFP expression in multiple sensory neurons of all segments, as well as in the epidermal cells of the posterior compartment (Fig. 5A). Older adults (1-7 days) largely lose the epidermal expression, but retain that in the sensory neurons. Although difficult to illustrate directly in the figures, it was apparent that the regions of epidermal GFP expression were usually more extensive than regions of sensory neuron expression - it was certainly not the case that all sensory hairs overlying a region of epidermis with GFP were innervated by GFP-expressing neurons.

In the coxa (Fig. 5B), the only GFP expression noted in sensory neurons was in those of CoHP3, one of three hair plates at the proximal end (Merritt and Murphey, 1992). In the trochanter, two clusters of large GFP-expressing sensory neurons were apparent; belonging to one of the hair plates (TrHP5), and to one of the fields of campaniform sensilla (TrCS5) (nomenclature according to Merritt and Murphey, 1992) (Fig. 5C). At the proximal end of the femur, *en*-driven GFP was seen in the ventral field of campaniform sensilla (Fig. 5C). Interestingly, GFP was only expressed in 7 out of the 11 neurons innervating this field.

The interior of the femur in the region of the femoral chordotonal organ was examined closely for traces of GFP expression, however, none was apparent. Although a large patch of GFP-expressing epidermal cells covered the posterior face of the femur (Fig. 5A), none of the many large tactile sensory bristle sensilla in that region showed signs of fluorescence. Towards the distal end of the femur was an elongated neuron that is probably a stretch receptor (Fig. 5D).

At the proximal end of the tibia, many of the hairs were innervated by single small neurons that expressed GFP. Also visible were large neurons innervating the single campaniform sensilla (Fig. 5D), and a few strongly fluorescent neurons that appeared to innervate hairs that were curved and bractless. These may be multimodal sensory neurons (Murphey et al., 1989a). At the distal end of the tibia were several large mechanosensory bristles, singly innervated by small GFP-expressing neurons (b in Fig. 5E), and clusters of brightly stained neurons that innervated curved hairs, again, probably multimodal hairs.

There were many *en*-driven GFP-expressing neurons in the tarsal segments (Fig. 5F), some singly innervating mechanosensory bristles, and others in clusters, innervating what may be multimodal sensory hairs. At the distal end of the tarsomeres were large, less fluorescent neurons that are probably stretch receptors (Smith and Shepherd, 1996) (Fig. 5F).

In the CNS, *en*-driven GFP expression was seen in afferents that arborized throughout the leg neuropil (Fig. 6 A-C), in a pattern identical to that described previously using transmitted light microscopy (Williams et al., 2000). Using the classification of neuropil subdivisions described for *Drosophila* and *Phormia* (Merritt and Murphey, 1992, Murphey et al., 1989a), leg afferents were seen to arborize in the ventral and intermediate association centers (Fig. 6A), consistent with their arising from tactile and/or gustatory afferents. More dorsally, leg afferents branched around the leg neuropil in the anterior and posterior lateral association centers, (Fig. 6B), probably corresponding to hair plate afferents. Other afferents projected through the oblique tract towards the midline, a group of which went on to project intersegmentally (Fig. 6C), a characteristic of type C campaniform sensilla afferents (Merritt and Murphey, 1992, Murphey et al., 1989a). Dorsal to this were a tight cluster of afferents that arose in the oblique tract, turned dorsally, then branched horizontally to project laterally and medially (Fig. 6C). The medial branch of these afferents appeared to cross the midline then join with the intersegmental campaniform tract. This unique type of projection is similar to that of trochanteral campaniform sensilla described for *Phormia* (Merritt and Murphey, 1992). All these types of leg afferent projections were visible in the mesothoracic and metathoracic neuromeres, although their relative positions were distorted.

No obvious *en*-driven GFP in wing sensilla

Despite some *en*-driven GFP fluorescence being present in the posterior portion of the wings shortly after eclosion (Fig. 1), no GFP-expressing sensory neurons were observed and this was confirmed by the absence of fluorescent afferents entering the CNS via the anterior dorsal mesothoracic nerve (ADMN). However, the ADMN, as did other nerves, often exhibited large GFP-expressing glial cells (Fig. 6B, C, D).

En-driven GFP in haltere sensory afferents

In the haltere, *en*-driven GFP was expressed in the posterior region, throughout the epidermis (Fig. 7A). It was also expressed in two populations of sensory neurons; a subset of the chordotonal neurons in the scabellum (Fig. 7B) and in all 5 of the large bristle sensory neurons on the ventral capitellum (Fig. 7C). GFP was absent from campaniform sensillar neurons that comprise most of the haltere sense organs, and from other bristle afferents in the posterior compartment of the capitellum. The GFP-expressing haltere afferents were particularly conspicuous in the dorsal part of the CNS (Fig. 6D), extending typical medial and lateral tufts (Trimarchi and Murphey, 1997) and projecting anteriorly to the subesophageal ganglion via the cervical connectives. The bristle afferents from the capitellum extended their arbors more ventrally in the metathoracic neuromere (Fig. 7C, D).

En-driven GFP in abdominal sensory afferents

Only small amounts of *en*-driven GFP fluorescence are visible in low resolution images in abdominal segments, corresponding to thin strips of epidermis at the posterior of each segment, as described previously (Hama et al., 1990) (Fig. 1). However, at higher resolution, a single GFP-expressing dendritic arborization (da) sensory neuron is visible in the sternal region of each segment (Fig. 7D). The cell bodies of these neurons are located more laterally, often associated with the tracheae, and extend neurites ventrally and medially to form an extensive arborization over the dorsal surface of the ventral muscles. These dendrites have numerous varicosities (Fig. 7D). The axons of these sensory neurons are visible entering the abdominal neuromeres of the CNS, although their arborizations are not readily distinguishable from GFP-

expressing interneurons and thus could not be traced with any certainty (Fig. 6D). These abdominal multidendritic da neurons have a similar position and dendritic morphology to a previously described abdominal sensory neuron (Shepherd and Smith, 1996, Smith and Shepherd, 1996) that was identified as v'pda (Williams and Shepherd, 1999), although more recent data on cell movements during metamorphosis suggest that it may in fact be vpda (Darren Williams, personal communication).

GFP fluorescence is absent from sensory cells of Wheeler's Organ. Small numbers of GFP-expressing tactile sensilla are present in the genital regions of both male and female (Fig. 7E) but these were not described in detail.

Discussion

Does GFP expression accurately reflect that of Engrailed?

The e16E *en-GAL4* insertion is thought to be in or near the *en* gene itself, and to express GAL4 in a pattern similar or identical to that of *en*, at least in the embryo (Yoffe et al., 1995). Little information is available regarding the similarity of its expression to that of *en* in the adult insect. Additional differences in temporal and spatial expression patterns could arise from a delay in synthesis and greater stability of the membrane-linked GFP reporter construct. It was therefore important to compare *en-GAL4*-driven mCD8::GFP expression to that of the Engrailed protein using the 4D9 antibody. These experiments showed a generally good correspondence in qualitative localization between 4D9 and GFP; all GFP-expressing cells contained some 4D9 staining, albeit faint in some cases. Not all 4D9-stained cells contained GFP, but this is most likely because the antibody also recognizes the En paralogue, Invested (Patel et al., 1989b). However, there was often poor correspondence between the intensity of GFP and antibody staining - intensely GFP-fluorescent neurons could exhibit relatively faint 4D9 immunofluorescence. These differences are probably due to differences in perdurance of the En protein versus the GAL4 and mCD8::GFP proteins. GFP is rather stable, with a half-life of approximately 10 h (Li et al., 1998). In this case, the strong GFP expression would be indicative of previously higher *en* gene activity that subsequently declined. Overall, the 4D9 staining does support the assumption that *en-GAL4*-driven mCD8::GFP expression does accurately label *en*-expressing neurons.

Engrailed expression in posterior auditory neurons

Engrailed expression in auditory neurons of Johnston's organ (JO) is confined almost completely to the posterior group. The detailed structure of the funiculus-pedicle joint and the insertions of the two groups of chordotonal neurons suggests that they are stimulated by sound in an alternating manner, with air movement from front to rear stretching the posterior group, and movements from rear to front stretching the medial group (Gopfert and Robert, 2002). This would partially account for the complexity of auditory responses recorded from the base of the antennae (Eberl et al., 2000). The circumstantial evidence from the present study suggests that differential En expression could distinguish the two groups. Whether it correspondingly influences the arborizations and synaptic outputs of the auditory afferents remains to be determined, however, there was no obvious correspondence with any particular zone of afferent projections defined previously (Kamikouchi et al., 2006).

En protein is present in the posterior part of the antennal disc in the late third instar (Raftery et al., 1991), while the JO neurons appear between 3 and 5 h after puparium formation (APF) in three clusters (Lienhard and Stocker, 1991). At least one of these clusters (j2) falls within the En-expressing region, so it is possible that it goes on to found the posterior group of En-expressing JO neurons.

Engrailed expression in subsets of olfactory neurons is not restricted to the posterior compartment

The olfactory sensilla of the funiculus are specified in the first 10 h APF, while fully differentiated neurons appear at 22 h APF (Reddy et al., 1997). These sensilla arise in both anterior and posterior sides of the imaginal disc (Reddy et al., 1997), and the present study shows that neurons on all sides of the adult funiculus and maxillary palp can express En. It is clear that the olfactory neurons that arise from the anterior compartment must express En *de novo* rather than inherit it from their epidermal precursors.

The transcription factors that control olfactory sensillum type are known: Atonal specifies the formation of the coeloconic type of olfactory sensillum (Jhaveri et al., 2000), whereas Amos is required for the other types (Goulding et al., 2000). Lozenge (Lz), a Runt domain-containing transcription factor, regulates the amount of Amos; high levels of Lz result in basiconic sensilla, low levels in trichoid sensilla (Gupta et al., 1998). Less is known about the transcriptional control of olfactory receptor expression and the correlated projection to receptor-specific glomeruli. So far, only Lz and Acj6 have been implicated in these choices (Ray et al., 2007), with Notch signaling also playing a role (Endo et al., 2007). The results of the present study, coupled with the fact that Or22c, Or23a, Or42b, and Or83c have all been identified in a screen of En-binding targets (Solano et al., 2003), suggests that En may play a part in the combinatorial system of Or receptor choice. In this case, En would be more likely to have a repressive role, since *en*-driven GFP is not present in glomeruli correlated with any of these receptor molecules.

Interestingly, Acj6, a POU domain transcription factor, is also a putative target of En binding (Solano et al., 2003), and Acj6 has been shown to regulate synaptic targeting (Certel et al., 2000, Komiyama et al., 2004) and to activate the expression of choline acetyltransferase (Lee and Salvaterra, 2002), yet another target of En binding. The present study shows colocalization between *en*-driven GFP and Acj6 protein, suggesting that, if En has an effect on *acj6* transcription, it cannot be repressive. Similarly, olfactory neurons are cholinergic (Lee and Salvaterra, 2002) so En also cannot be acting as a repressor of choline acetyltransferase.

Engrailed in the labial palps correlates with position, not modality

Engrailed expression in the labial palps of the proboscis appears to correspond exactly to the location of the posterior compartment, including the exclusion of the inner surface of the palp from this region (Yasunaga et al., 2006). All neurons in all sensillum types seem to be labeled, including the few purely mechanosensory bristles. So in this structure, in contrast to the olfactory neurons of the antenna and maxillary palps, En expression appears to correlate with sensillum position and does not distinguish sensilla of different modalities. Individual gustatory neurons within a sensillum express different gustatory receptors and respond to different taste qualities, and may project to different regions of the subesophageal neuropil (Wang et al., 2004). En is probably not involved in controlling this process, since there are no antero-posterior correlates of gustatory receptor distribution. However, there is also a segregation of gustatory afferent projections based on their location in the periphery, for example, neurons with cell bodies in the proboscis project to different regions of the gustatory neuropil compared to those in the legs (Wang et al., 2004), so it is possible that there is a higher-resolution segregation based on anterior-posterior position within those organs, in which differential En expression could play a part.

En is expressed in subsets of posterior leg and haltere neurons

No sensory neurons expressing *en*-driven GFP were observed in the anterior regions of the legs. However, unlike the labial palps, only a subset of the sensory bristles in the posterior compartment expressed En. For example in the posterior region of the femur, there were large expanses of GFP-expressing epidermis, with many large mechanosensory bristle sensilla, none

of which expressed *en*-driven GFP. *En* expression does not distinguish between modalities, since neurons in campaniform sensilla, hair plates, stretch receptors, multimodal hairs and mechanosensory bristles all were observed expressing GFP. In certain organs such as hair plates or campaniform sensilla, only a portion of the sensillum field expressed *En*, even though the whole organ appeared to be located in the posterior compartment. There thus appears to be an uncoupling of *En*'s early role in specifying compartmental identity in the leg disc (Kornberg, 1981, Lawrence and Struhl, 1982, Morata and Lawrence, 1975) from its later role in determining the identity of sensory neurons. It was shown previously that compartmental identity does not determine the axonal projection (Murphey et al., 1989b), but the uncoupling of neuronal *En* expression from compartmental identity leaves open the possibility that *En* could be involved in the control of sensory axon guidance, as is known to be the case in cockroach sensory neurons (Marie et al., 2000).

Similarly results were found for the haltere, where *En* expression is restricted to the posterior compartment, but only in a subset of the sensory neurons within it. It is not clear if, or how, the *En*-expressing subset of haltere chordotonal organs can be functionally distinct from the other, unless perhaps they respond to different frequencies of movement.

A single abdominal md neuron expresses *En*

The adult *En*-expressing md neuron was tentatively identified as either v'pda or vpda, based on its marked similarity to a persistent larval neuron (Shepherd and Smith, 1996, Smith and Shepherd, 1996, Williams and Shepherd, 1999). In either case there have been no reports of v'pda or vpda expressing *En* in the larva; the neurons that do are the lch5 neurons, dmd1 and lbd (Brewster et al., 2001). The lateral bipolar dendrite neuron, lbd, is also persistent (Williams and Shepherd, 1999) but, unless it has undergone unprecedented migration and dendritic remodeling, is unlikely to be the neuron observed in this study. It is more likely, therefore, that the abdominal md neuron v'pda/vpda expresses *En de novo*. The function of this later *En* expression, however, remains obscure.

What is the function of *En* expression in the adult?

The observations discussed above show that the correlation of posterior compartment location to *En* expression ranges from a complete one, in case of the labial palp and perhaps the pedicel of the antenna, through a partial correlation (*En* expression in a subset of posterior neurons in the leg and haltere), to no correlation, in the case of the olfactory neurons and abdominal md neurons. Thus the role of *En* expression is likely to be different in different organs, and perhaps between different sensory cell types in the same organ. During pupal development, the role of *En* could range from helping determine the complement of olfactory receptors, to contributing to the regulation of somatotopic differences in axonal projections and synaptic connectivity, as was shown for cockroach cercal sensory neurons (Marie et al., 2002).

These aspects of neuronal phenotype, however, are established by the time of eclosion, and have not been shown to vary during adult life. We have seen that *en*-driven membranous GFP persists in neurons of 1 week old adults, and a quantitative study of *en-lacZ* expression in the adult antenna determined that levels of expression fall only to about 60% of their initial levels over the entire lifetime of the animal (Rogina and Helfand, 1997). In other, more long-lived, insects it has been shown that *En* protein is persistently expressed in the adult CNS and sensory neurons (Blagburn et al., 1995, Siegler et al., 2001). What is the role of the persistent *En* expression seen in adult sensory neurons? It could be required to maintain expression of the appropriate complement of olfactory receptors or axonal and synaptic adhesion molecules, but this could be accomplished as effectively by, for example, modifying histones. In addition, we showed previously that knockout of *En* has no effect on those axonal branches and synaptic connections that have already formed (Marie et al., 2002), suggesting that, if those

characteristics are plastic, En is not required to maintain them. In mice, the En paralogues En1 and En2 are required for postembryonic dopaminergic neuronal survival (Simon et al., 2001), with partial knockouts showing a slow progressive neuronal degeneration that resembles the pathology of Parkinson's disease (Sgado et al., 2006). Whether Engrailed plays a similar role in *Drosophila* neurons is an interesting question for future investigation.

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Figure 1. mCD8::GFP expression driven by *en-GAL4* in a 4h adult male. Strong GFP fluorescence (blue-green) is present in the antennae (a), the maxillary palps (b) and labial palps (c) of the proboscis, and in rings around the ocelli (d). In the thorax, GFP is expressed in all segments of the legs (e), in one direct flight muscle (f), in the wing hinge and posterior wing veins (g) and in the haltere (h). In the abdomen, GFP is expressed in stripes at the posterior of each segment (i), in the hindgut (j) and in the genitalia (k). Cuticular autofluorescence is shown in orange-red, with eye photochromes in yellow. Scale bar: 500 μ m.

Figure 2.

mCD8::GFP expression driven by *en-GAL4* in the antenna and proboscis. **A.** Anterior view of antenna of 4 h adult, GFP fluorescence in blue-green, cuticular autofluorescence in orange-red. The three segments, pedicel (Ped), funiculus (Fun) and arista (Ar) are indicated. **B.** GFP fluorescence and nuclear 4D9 immunostaining for En and Inv (magenta). *En*-driven GFP and En/Inv protein are present in a subset of Johnston's organ neurons (JO), in neurons near the base of the arista (Ar), and in many olfactory neurons (Olf) in the funiculus. The olfactory axons are indicated as they traverse the pedicel (arrow). Some En/Inv-positive cells in the pedicel wall do not express *en*-driven GFP (asterisk). All GFP-expressing olfactory neurons contain nuclear 4D9 staining, although a few cells are En/Inv-positive but appear not to express

GFP (arrowheads). **C.** Transverse slices through the pedicel show that GFP-expressing neurons are mainly confined to the posterior (post) group of the Johnston's organ, and are largely absent from the medial group (med). **D.** High power view of trichoid sensilla olfactory sensory neurons, stained with *en*-driven GFP (green) and 4D9 anti-En/Inv (red). **E.** Basiconic sensilla sensory neurons. Multiple dendrites innervate the sensilla. **F.** Frozen section of adult funiculus showing *en*-driven GFP (blue-green) and Acj6 immunoreactivity (magenta). Many neurons contain Acj6 alone (arrows), most GFP-expressing neurons contain Acj6 (asterisks). **G.** Lateral view of maxillary palp, showing *en*-driven GFP in olfactory neurons. **H.** Lateral view of labial palp, showing GFP expression under basiconic sensilla. Note that there is no expression in mechanosensory bristles (m). **I.** Higher magnification of posterior labial palp, with cuticle and pseudotracheae optically cut away to show GFP fluorescence in neurons innervating all posterior sensilla, including large (l) small (s) and intermediate (i) gustatory, and mechanosensory (m). Scale bar: 100 μ m in A, G and H; 50 μ m in B, C, F and I; 25 μ m in D and E.

Figure 3.

mCD8::GFP expression driven by *en-GAL4* in the brain. **A.** Anterior overview of the brain of a 4 h adult, GFP fluorescence in blue-green, 4D9 immunostaining for En and Inv in magenta. En is expressed in two clusters of neurons in the protocerebrum. **B.** Higher magnification of central region indicated in A. Non-sensory GFP fluorescence is shown in green, with false colors indicating the sensory projections: olfactory (blue), auditory (white), and pharyngeal nerve gustatory (magenta). A pair of large descending interneurons is indicated by asterisks. For clarity, much of the GFP-expressing circumoesophageal glia (arrowhead) have been optically removed. Scale bar: 100 μ m in A, 50 μ m in B.

Figure 4. *en*-driven GFP expression in identified olfactory glomeruli. **A.** Serial confocal Z-projections of slices through the olfactory lobes, counter-stained with nc82 Bruchpilot antibody. Projections are from the anterior, at 10 μ m intervals. **B-E.** 3D reconstructions of GFP-labeled glomeruli, moving from anterior (B) to posterior (E). Scale bar: 50 μ m in A, 25 μ m in B-E .

Figure 5. mCD8::GFP expression driven by *en-GAL4* in the prothoracic leg. GFP fluorescence is shown in blue-green, cuticular autofluorescence in orange-red. **A.** Posterior view of left prothoracic leg of a 4 hour adult. GFP expression in sensory neurons and in portions of the epidermis. **B.** GFP expression in 3 neurons of a hair plate (CoHP3) at the proximal end of the coxa. **C.** GFP in 5 neurons of a hair plate of the trochanter (TrHP5), 8 neurons of a campaniform sensillum of the trochanter (TrCS8), and in only 7 of 11 neurons of a campaniform sensillum at the proximal end of the femur (FeCS11). **D.** Femur-tibia joint, with GFP expression in a stretch receptor neuron (SR) in the femur, in a solitary campaniform sensillum in the tibia (TiCSv1) and in a sensory neuron innervating a curved, bractless hair (m). **E.** Distal tibia. GFP in neurons

singly innervating straight hairs (b) and in clusters of neurons innervating curved hairs (m).

F. First tarsomere. GFP expression in neurons singly innervating straight hairs (b), in clusters of neurons innervating curved hairs (m), and also in a stretch receptor neuron (SR) at the distal end. Scale bar: 200 μm in A, 50 μm in B-F.

Figure 6.

en-driven GFP expression in the CNS. Z-projections of confocal slices through the ganglia, moving from ventral (A) to dorsal (D). Anterior to the top. Axons of leg sensory neurons are highlighted in white, those of the haltere are highlighted in blue. White bars indicate where nerves have been trimmed from the image. **A.** In the prothoracic neuromere axons in the ventral association center are indicated (arrows). Midline clusters of GFP-expressing neurons are indicated (asterisks) **B.** In the prothoracic neuromere axons in the anterior lateral association center (a) and oblique tract (o) are indicated. GFP-expressing glia are also indicated (arrowheads). **C.** More dorsal in the prothoracic neuromere are obliquely projecting leg afferents (o) and intersegmental projections (s). GFP-expressing glia are also indicated (arrowheads). **D.** Haltere afferents shown in blue. Lateral (l) and medial (m) tufts are indicated, as is the putative capitellum bristle projection (b). GFP-expressing glia in the connective and ADMN are indicated by arrowheads, abdominal sensory afferents by double arrowheads, and the large descending interneuron axons by asterisks. Scale bar: 100 μ m.

Figure 7.

mCD8::GFP expression driven by *en-GAL4* in the haltere and abdomen. GFP fluorescence shown in blue-green, tissue autofluorescence in orange-red. **A.** Overview of right haltere of 4h adult, viewed from ventral, proximal to top of picture. GFP is present in the posterior epidermis and notably in chordotonal sensory neurons (ch) of the scabellum, and in ventral bristle sensory neurons (br) of the capitellum. Little GFP is associated with campaniform fields. **B.** High power transverse projection of scabellar chordotonal neurons (asterisk) retrogradely marked with dil (magenta), a distal subset of which are GFP-fluorescent. Campaniform sensilla sensory neurons (arrowheads) do not express GFP. **C.** High power view of the bristles on the capitellum, with some cuticle graphically removed to reveal the underlying GFP-fluorescent sensory neurons.

D. Interior view of right abdominal hemisegments of 4h adult female, viewed from dorsal. Anterior to top, lateral to right. A single GFP-expressing multidendritic sensory neuron is visible in each segment. **E.** Dorsal view of the female genitalia, showing two small bristle sensory neurons.

Scale bar in A: 50 μm in A, 25 μm in B, 20 μm in C. Scale bar in D: 100 μm . Scale bar in E: 50 μm .

en-driven mCD8::GFP expression in antennal lobe glomeruli. Fluorescent glomeruli and their corresponding olfactory receptors are indicated in bold.

Table 1

Name	Type	Shape	Or expression [single cell]	Glomeruli	En-GFP
ab1	ant. basiconic	large	[10a] [42b] [92a] [Gr21a, Gr63a]	DL1, DM1, VA2, V	No
ab2	ant. basiconic	large	[59b] [85a]	DM4, DM5	No
ab3	ant. basiconic	large	[22a, 22b] [85b]	DM2, ?	No
ab4	ant. basiconic	thin	[7a] [56a]	DL5, DA2	Yes
ab5	ant. basiconic	thin	[47a] [82a]	DM3, VA6	Yes
ab6	ant. basiconic	thin	[49b] [85b, 98b]	VA5, VM5d?	Yes, not VM5d
ab7	ant. basiconic	small	[67c] [98a]	VC4/VC3m, VM5v	No
ab8	ant. basiconic	small	[9a] [43b]	VM3, VM2	Yes
ab9	ant. basiconic	small	[67b] [69aA, 69aB]	VA3, D	No
ab10	ant. basiconic	small	[49a, 85f] [67a]	DL4, DM6	No
at1	Ant. trichoid	T1	[67d]	DA1	No
at2	Ant. trichoid	T2	[23a] [83c]	DA3, DC3	No
at3	Ant. trichoid	T3	[2a] [19a, 19b] [43a]	DA4m, DC1, DA4l	Yes, not DA4l
at4	Ant. trichoid	T3	[47b] [65a, 65b, 65c] [88a]	VA1v, DL3, VA1d	No
ac3	ant. coeloconic		[?] [35a]	VC3	No
ac	ant. coeloconic			DC4	Yes
ac	ant. coeloconic			DL2	No
ac	ant. coeloconic			VL1	No
ac	ant. coeloconic			VL2a	No
ac	ant. coeloconic			VM1	Yes
ac	ant. coeloconic			VM4	No
ac	ant. coeloconic			VM6	Yes
ai1	ant. intermed.		[13a]	DC2	Yes
pb1	max. palp basiconic	thin	[42a] [71a]	VM7, VC2	No
pb2	max. palp basiconic	thin	[35c, 85e] [46aA]	VC1, VA7l	Yes
pb3	max. palp basiconic	thin	[59c] [85d]	1, VA4	No