

Axial patterning of *C. elegans* male sensilla identities by selector genes

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Received for publication 11 September 2003, revised 20 January 2004, accepted 26 January 2004

Abstract

The fan and rays of the *C. elegans* male tail constitute a compound sensory organ essential for mating. Within this organ, the individual sensilla, known as rays, have unique identities. We show that ray identities are patterned by a selector gene mechanism in a manner similar to other serially homologous axial structures. One selector gene that promotes the identities of a subset of the rays is the Hox gene *egl-5*. Within EGL-5-expressing rays, further patterning is provided by a Pax-6 homolog and a signal of the TGF β family. These genes and pathway coordinately specify multiple ray properties affecting all three terminal ray cell types. These properties include complex patterns of FMRamide-like (FaRP) neuropeptides, serotonin (5HT) and dopamine expression, and ray morphology. Differences in these differentiated characteristics give each sensillum a unique identity and potentially endow the compound ray organ with a higher-order information gathering capacity.

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Keywords: *C. elegans*; Neuron subtype; Neurotransmitter; Dopamine; Serotonin; FMRamide; Hox; Pax-6; TGF β ; Male

Introduction

The genetic programs that invest neural cells with their diverse properties play a major role in shaping animal behavior, particularly in simple animals that exhibit little experience-dependent nervous system plasticity. Defining these developmental programs represents an essential step towards understanding how behavior is genetically encoded and how it evolves through changes in patterns of gene activity. For example, neuron diversification within a sensory organ allows a greater amount of information to be conveyed about the environment. Such higher-order information is essential to guide complex animal behaviors and it is thus of interest to consider how neuron differentiation within a sensory structure is genetically specified.

Differentiation of a spatially organized sensory system presents two problems: how are individual sensory cell types specified and how is each cell given a unique identity according to its position within the structure? We have studied these questions using as a simple model a small array of sensilla in the tail of the *C. elegans* male. The *C. elegans* male tail has a genital structure consisting of nine pairs of finger-like sensilla, known as rays, which protrude bilaterally within a cuticular fan (Sulston et al., 1980). The fan and rays allow the adult male to sense contact with the hermaphrodite and to determine his orientation and position with respect to the hermaphrodite body during mating (Liu and Sternberg, 1995).

The fan and rays appear to comprise a compound sensory organ in which the individual rays convey information both about a particular sensory stimulus and about the location where this stimulus is received. Each ray, a protrusion of the hypodermis, contains the dendritic processes of two ultra-structurally distinct sensory neurons, A-type (RnA) and B type (RnB). The dendritic endings of these neurons are held at an opening to the environment by a support cell known as the ray structural cell (Rnst) (Sulston et al., 1980). Thus, rays are similar to each other with respect to structure and cell composition. Rays, however, differ from one another in some respects. They are located at stereotyped, genetically determined positions in the fan and they have distinct

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morphologies and molecular properties, including different neurotransmitter and receptor expression patterns (Lints and Emmons, 1999; Loer and Kenyon, 1993; Schinkmann and Li, 1992; Sulston and Horvitz, 1977; Sulston et al., 1980; Troemel et al., 1995). Recently, we have determined that the RnA and RnB neurons of different rays synapse onto different subsets of target neurons in the preanal ganglion (S. W. E., D. Albertson, D. H. Hall, and H. Eckholdt, unpublished data). Consistent with these observations, selective ablation studies reveal that loss of individual rays results in ray-specific effects on male copulatory behavior (Liu and Sternberg, 1995). These observations indicate that each ray is functionally specialized and suggest that the fan and rays convey complex topological information to guide the male in his mating attempt.

How is ray specialization genetically encoded? Previous studies have shown that properties common to all rays and properties that differ are specified by independent genetic programs. In all rays, the same sublineage is used to generate the three terminal cell types (Sulston and Horvitz, 1977; Sulston et al., 1980). The sublineage is initiated in a hypodermal stem cell known as the ray precursor cell (Rn) by the proneural bHLH transcription factors *lin-32* and *hlh-2*, respectively homologs of *atonal* and *E/daughterless* (Portman and Emmons, 2000; Zhao and Emmons, 1995).

As in other systems, the rays, as serially homologous structures, are differentiated from each other by selector genes, which cause a subset of properties to differ from those of a ground state (Casares and Mann, 2001; Garcia-Bellido, 1975; Mann and Morata, 2000; Waskiewicz et al., 2002; Weatherbee and Carroll, 1999). Our earlier studies and those of others showed that the *C. elegans* Hox genes *mab-5*, a member of the *ftz* paralog gene family, and *egl-5*, a member of the *Abdominal-B* paralog gene family (Aboobaker and Blaxter, 2003), appear to act as selectors of ray identity. Loss-of-function and gain-of-function mutations in these genes resulted, respectively, in posterior-to-anterior and anterior-to-posterior morphological transformations and ray fusions. Ray fusions suggest that changes in ray identity are accompanied by changes in the cell-association properties of the Rnst cells (Baird et al., 1991; Chisholm, 1991; Chow and Emmons, 1994; Hahn and Emmons, 2003; Salser and Kenyon, 1996). These studies, however, did not address the fate of RnA and RnB neurons in affected rays.

Previously, we demonstrated that *egl-5* is required not only for the morphology of rays 3–5 but also for competence of their RnA neurons to adopt a dopaminergic fate (Lints and Emmons, 1999). Here, we show that *egl-5* is also necessary for the characteristic expression of serotonin (5HT) and a complex profile of FMRFamide-like (FaRP) neuropeptides in RnB neurons. Hence, *egl-5* determines properties of all three terminal branches of the ray sublineage. Ray identities among EGL-5-expressing rays (rays 3–6) are further refined by a TGF β -like signaling pathway and by the *Pax-6*-like gene *mab-18*. These modifiers

differentiate rays 5 and 6 from the most anterior EGL-5-expressing ray, ray 3. Further, we show that loss of *egl-5* function results in a wholesale homeotic transformation of the properties of rays 3–5 to those of the more anterior ray 2, which does not express *egl-5*. These data suggest that the wild-type patterns of ray 2–6 identities are derived through the progressive anteroposterior refinement of an *egl-5(-)* ground state identity.

Materials and methods

C. elegans strains and cultures

Nematodes were grown and maintained as described in Brenner (1974). Bristol (N2)-derived strains *him-8(e1489)* and *him-5(e1490)*, which generate a high incidence of males, were used as reference wild-type strains. Unless otherwise stated, strains were maintained at 20–25°C. The following genotypes were used.

Mutant alleles: LG II: *sma-6(wk7)*; LG III: *egl-5(u202)*, *daf-4(m592ts)*, *sma-2(e502)sma-3(e491)* and *sma-2(e502)sma-3(e491)sma-4(e729)* (courtesy of C. Savage-Dunn), *sma-2(e502)*, *sma-3(e491)*, *sma-4(e729)*, *pha-1(e2123ts)*; LGIV: *him-8(e1489)*; LGV: *dbl-1(wk70)*, *him-5(e1490)*; LGX: *mab-18(bx23)*. All mutant strains have been described previously (Baird et al., 1991; Brenner, 1974; Chisholm, 1991; Granato et al., 1994; Krishna et al., 1999; Savage et al., 1996; Suzuki et al., 1999; Wang et al., 1993; Zhang and Emmons, 1995; consult also Riddle and Albert, 1997). *sma-6(wk7)*, *dbl-1(wk70)* and *egl-5(u202)* are presumptive null mutations (Krishna et al., 1999; Suzuki et al., 1999; Wang et al., 1993).

Chromosomally integrated transgenic arrays: *Is[tph-1::GFP, pRF4]V* (Sze et al., 2000); *bxIs16V*, [*tph-1::CFP* (EM#310), *cat-2::YFP* (EM#303), pBX-1]; *bxIs14V*, [*pkd-2::GFP*, pBX-1] (L. J. and S.W.E., unpublished data; Barr and Sternberg, 1999); *ynIs24V*, [*flp-5::GFP*]; *ynIs67III*, [*flp-6::GFP*]; *ynIs46*, [*flp-11::GFP*]; *ynIs64I*, [*flp-17::GFP*] (K. K. and C. L., unpublished data).

Extrachromosomal transgenic arrays: *bxEx70* and *bxEx71*, are independently generated duplicate versions of a complex extrachromosomal array consisting of *Ex[hs::dbl-1(pMYHSdbl-1), tph-1::CFP* (EM#310), *cat-2::YFP* (EM#303), N2 DNA, pBX-1]; *bxEx72* is identical to *bxEx70* and *bxEx71* except that pMYHSdbl-1 is replaced with empty heat-shock vector pPD49.78. Co-transformation marker pBX-1, which contains *pha-1(+)*, was used in *pha-1(e2123ts)* backgrounds (Granato et al., 1994); *bxEx83* is a complex array containing *mab-23::GFP* (Lints and Emmons, 2002).

Serotonin- and FaRP-positive ray neuron cell identification

Expression of serotonergic fate by ray neurons was determined using *tph-1* reporters and by staining with

Table 1
Summary of neurotransmitter patterns in wild-type, mutant and *hs::dbl-1*-treated animals

<i>wild type</i>									
Transmitter (neuron)	1	2	3	Ray 4	5	6	7	8	9
DA (A)					+		+		+
5HT (B)	+		+						+
FLP-X (B)		+							
<i>flp-5</i> (B)	+				+		+		
<i>flp-6</i> (B)		+			+	+	+		
<i>flp-11</i> (?)				+					
<i>flp-17</i> (B)	+				+		+		

<i>egl-5(0)</i>									
Transmitter (neuron)	1	2	3	Ray 4	5	6	7	8	9
DA (A)									+
5HT (B)	+								75%
FLP-X (B)		+	+	+	+				
<i>flp-5</i> (B)	+								+
<i>flp-6</i> (B)		+	+	+	+				+
<i>flp-11</i> (?)									
<i>flp-17</i> (B)	+								+

<i>dbl-1(0) pathway</i>									
Transmitter (neuron)	1	2	3	Ray 4	5	6	7	8	9
DA (A)					10%		30%		
5HT (B)	+		+		+				
FLP-X (B)		+							
<i>flp-5</i> (B)	+								
<i>flp-6</i> (B)		+				+	+		
<i>flp-11</i> (?)				+					
<i>flp-17</i> (B)	+								

<i>egl-5(0); dbl-1(0) pathway</i>									
Transmitter (neuron)	1	2	3	Ray 4	5	6	7	8	9
DA (A)									
5HT (B)	+								
FLP-X (B)		+	+	+	+				
<i>flp-5</i> (B)	+								
<i>flp-6</i> (B)		+	+	+	+				
<i>flp-11</i> (?)									
<i>flp-17</i> (B)	+								

<i>egl-5(0); mab-18(lf)</i>									
Transmitter (neuron)	1	2	3	Ray 4	5	6	7	8	9
DA (A)									+
5HT (B)	+								70%
FLP-X (B)		+							
<i>flp-5</i> (B)	+								+
<i>flp-6</i> (B)		+	+	+	+				+
<i>flp-11</i> (?)									4%
<i>flp-17</i> (B)	+								+

<i>dbl-1(0) pathway; mab-18(lf)</i>									
Transmitter (neuron)	1	2	3	Ray 4	5	6	7	8	9
DA (A)									
5HT (B)	+								
FLP-X (B)		+							
<i>flp-5</i> (B)	+								5%
<i>flp-6</i> (B)		+							50%
<i>flp-11</i> (?)				+					
<i>flp-17</i> (B)	+								

<i>hs::dbl-1; dbl-1(0) pathway</i>									
Treatment: Transmitter	1	2	3	Ray 4	5	6	7	8	9
No heat-shock:									
DA (A)									6%
5HT (B)	+								20%
+heat shock:									
DA (A)				40%	55%	+	10%	+	10%
5HT (B)	+			50%	5%		2%		5%

<i>hs::dbl-1; dbl-1(0) pathway; mab-18(lf)</i>									
Treatment: Transmitter	1	2	3	Ray 4	5	6	7	8	9
No heat-shock:									
DA (A)									8%
5HT (B)	+			+			+	+	
+heat shock:									
DA (A)				38%	60%	+	10%	+	20%
5HT (B)	+			52%	5%		+		2%

+, neurotransmitter marker expression observed in 100% of male sides; for markers expressed at frequencies <100%, frequency is shown. Ray neuron type, rNA(A) or rNB(B), that expresses neurotransmitter, is indicated. Rays descended from seam cell V6 (rays 2–6) are highlighted by the grey box. For *dbl-1* pathway mutants, frequencies shown are representative of all single pathway mutants, double and triple mutant combinations tested (see Materials and methods, Fig. 3 legend). +heat-shock treatment = 30 °C, 30 min at Rn stage. *n* (number of sides scored per genotype per treatment) = 30–150. DA, dopamine; 5HT, serotonin.

anti-5HT antibodies (purchased from H. W. M. Steinbusch, Maastricht University, The Netherlands) as described by Loer and Kenyon (1993). In all genetic backgrounds examined, the *tph-1* reporter expression pattern and 5HT antibody staining were consistent. FLP-X FMRF-amide fate expression was determined by staining with anti-FMRF-amide antibodies as described by Schinkmann and Li (1992). Assignment of 5HT or FMRF-amide antibody-positive fate to specific RnB neurons was done by performing antibody staining on animals carrying RnB- or RnA-specific reporters: *pkd-2::GFP* (*bxIs14*, a marker for all RnB neurons except R6B), *cat-2::YFP/tph-1::CFP* (*bxIs16*, markers for R5A, R7A, R9A and R1B, R3B and R9B, respectively) or *mab-23::GFP* (*bxEx83*, a marker for R1A–R4A and R6A nuclei). Assignment of *flp-5*, *flp-6* and *flp-17::GFP* reporter expression to the RnB neurons was done by examining the F1 male cross progeny of *flp* reporter strains mated with strains carrying either *bxIs16* or *bxEx83* and by staining *flp* reporter strains directly with anti-5HT antisera (which labels R1B, R3B and R9B) or anti-FMRF-amide antisera (which labels R2B). *flp* reporter expression patterns overlapped only with RnB neuron but not RnA neuron markers ($n > 20$ male sides examined for each co-labeling strategy).

Generation of transgenic animals

Transgenic nematodes were generated by microinjection of DNA as described by Mello et al. (1991). Transgenic lines carrying extrachromosomal transgenic arrays *bxEx70* and *bxEx71* were generated by co-injection of linearized blunt-ended fragments of pMYHSdbl-1 (0.5–1 ng/μl), *cat-2::YFP* EM#303 (1 ng/μl), *tph-1::CFP* EM#310 (6 ng/μl), *pha-1(+)* pBX-1 (1 ng/μl) and *C. elegans* genomic DNA (75 ng/μl) into a *pha-1(e2123ts); him-5(e1490)* strain (Granato et al., 1994). pMYHSdbl-1 contains a *dbl-1* cDNA placed under control of the heat-shock promoter in vector pPD49.78 (courtesy of M. Yandell and W.B. Wood, University of Colorado, Boulder, CO; Stringham et al., 1992). EM#310 is a CFP derivative of the *tph-1::GFP* reporter described in Sze et al. (2000) and EM#303 is a YFP derivative of *cat-2::GFP* plasmid EM#282 (Lints and Emmons, 1999). Control array *bxEx72* was constructed similarly, substituting pMYHSdbl-1 with empty heat-shock vector pPD49.78 (a gift from A. Fire).

Heat shock and temperature shift experiments

Heat shock (Figs. 4C, F; Table 1) and temperature shift experiments (Figs. 4A–E; Table 1) were performed as described in Lints and Emmons (1999). For the heat-shock experiments, *dbl-1(wk70)*; *pha-1(e2123ts)*; *him-8(e1489)* or *dbl-1(wk70)*; *pha-1(e2123ts)*; *him-8(e1489)*; *mab-18(bx23)* strains carrying either *bxEx70*, *bxEx71* or *bxEx72* were used. In the temperature shift experiments, a *daf-4(m592ts)* strain

carrying *bxIs16* was used. In selected experiments, animals were examined for serotonergic or dopaminergic fate expression by staining with 5HT antisera or by performing formaldehyde-induced fluorescence (FIF) as described in Sawin et al. (2000), respectively (data not shown).

Results

Serotonin is synthesized by the RnB neurons of three rays

The ontogeny of the rays and their structure is summarized in Figs. 1A–C. Three of the nine rays, rays 1, 3 and 9, contain a single neuron that shows serotonin-like immunoreactivity (5HT-LI; Loer and Kenyon, 1993) and that expresses reporters for the gene encoding the 5HT biosynthetic enzyme tryptophan hydroxylase, *tph-1* (Sze et al., 2000). To determine whether these serotonergic neurons correspond to the RnA or the RnB neurons of rays 1, 3 and 9, we performed 5HT antibody staining experiments on transgenic strains expressing known RnA- or RnB-specific reporters (Materials and methods). RnA-specific markers used were *C. elegans* tyrosine hydroxylase gene *cat-2* (expressed in dopaminergic neurons R5A, R7A and R9A) and DM-domain transcription factor gene *mab-23* (expressed in R1A to R4A and R6A nuclei); RnB neurons were visualized with reporters for the putative calcium channel gene *pkd-2* (in all RnB neurons except R6B) (Barr and Sternberg, 1999; Lints and Emmons, 1999, 2002). In these experiments, the 5HT immunoreactivity co-localized with RnB, and not RnA, neuron markers indicating that the 5HT neurons present in rays 1, 3 and 9 correspond to R1B, R3B and R9B, respectively (Figs. 1D–F, 2A; Table 1).

Multiple FaRP neuropeptides are expressed in the RnB neurons

The *C. elegans* genome contains at least 22 genes that encode FMRFamide and related FaRP neuropeptides (*flp* genes); a total of 59 FaRPs are encoded by these *flp* genes (Li et al., 1999a,b; Nelson et al., 1998). To determine whether any ray neurons express FaRPs, we examined animals carrying transcriptional fusion reporter genes for 18 of the *flp* genes. In addition, we stained worms with an anti-FMRFamide antiserum that localizes to cells that do not express any of the transcriptional reporter constructs. Although we recognize that transcriptional reporter fusions sometimes do not accurately reflect the endogenous expression pattern of a gene, for this paper, we designate the peptide that reacts with this antiserum in the nonoverlapping cells as FLP-X. We found that FLP-X and reporters for *flp-5*, *flp-6*, *flp-11* and *flp-17* showed robust and reproducible expression in specific subsets of rays (Table 1; Fig. 1J; M. Barr, personal communication). For all genes except *flp-11*, we were able to determine that these FaRP-encoding genes

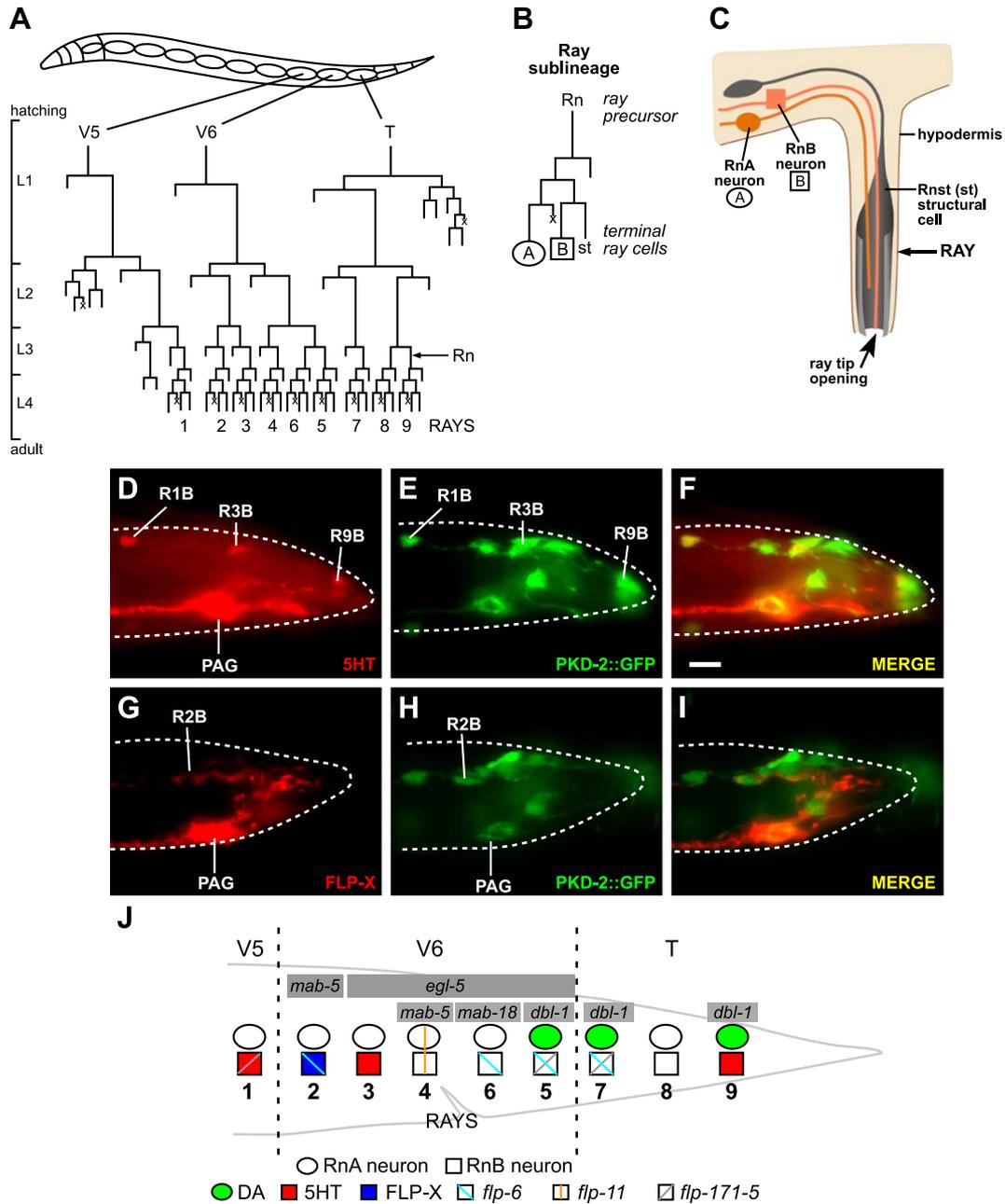


Fig. 1. Ray development and neurotransmitter patterning. (A) Postembryonic cell lineages leading to the ray neurons (Sulston and Horvitz, 1977). The scale to the left indicates larval stage (L). V5, V6 and T are hypodermal blast cells present in the lateral seam on each side of the animal at L1. These cells generate R1–R9, the ray precursor cells (Rn, where n stand for rays 1–9), by L3. (B) Rn cells divide by a stereotyped lineage pattern (the ray sublineage) to produce an RnA neuron (A, circle), an RnB neuron (B, square), an Rnst structural cell (st), and a programmed cell death (x) by the adult stage (Sulston and Horvitz, 1977). (C) Schematic of an adult male ray (lateral view). RnA, RnB and Rnst cell bodies are in the lumbar ganglia and their dendritic processes extend into the ray. RnA and RnB axons project anteriorly to targets in the preanal ganglia. (D–I) Fluorescent micrographs showing co-localization of 5HT (D–F) and FLP-X (G–I) immunoreactivity with RnB neuron marker PKD-2::GFP (lateral view). VNC, ventral nerve cord; PAG, preanal ganglion. Body outlined with grey dotted line. Scale bar = 10 μ m. (J) Summary of wild-type ray neurotransmitter expression patterns. Each ray contains an RnA (circle) and RnB (square) neuron. The color key below indicates the neurotransmitter markers expressed in neurons (Sulston and Horvitz, 1977, this study). *flp-11*::GFP is expressed weakly in several rays (not shown) but only strongly in one of the two neurons of ray 4. Vertical dotted lines divide rays on the basis of their L1 blast cell origin (V5, V6 or T). Grey bars above the ray neurons span those rays that express or whose morphologies are influenced by the regulatory gene indicated in the bar. DA, dopamine; 5HT, serotonin.

were expressed in RnB neurons by performing double labeling experiments with known RnA and RnB neuron markers (see Materials and methods) (FLP-X, Figs. 1G–I;

flp-5, *flp-6*, *flp-11*, *flp-17*, data not shown; Table 1). In the case of *flp-11*, expression of the reporter in several non-ray cells near the ray 4 neuron and in the overlaying hypodermis

prevented us from identifying which of the two ray 4 neurons expressed this reporter.

The pattern of neurotransmitter expression in the rays is remarkably complex (Table 1; Fig. 1J). Individual RnB neurons can express 5HT alone, one to three FaRP-encoding genes (e.g., neurons R1B, R5B, R7B) or FaRPs in combination with 5HT (e.g., R1B). The possibility that several *flp* genes may produce more than one type of FaRP adds further complexity to the repertoire of neurotransmitters potentially produced by a single ray neuron. With the exception of *flp-5* and *flp-17*, none of the seven neurotransmitters have identical expression patterns. Conversely, with the exception of rays 5 and 7, no two rays express the same combination of neurotransmitters. These RnB neuron neurotransmitter phenotypes, together with differential expression of dopaminergic fate among RnA neurons, contribute significantly to the expression of ray-specific identities.

Hox gene egl-5 is required for rays 3–6 identities and suppresses ray 2 identity

To understand how such complex patterns of neurotransmitter expression are genetically programmed and the extent to which different aspects of ray identity are coordinately controlled, we examined expression of 5HT and FaRP markers in mutants for regulatory genes known to define other ray characteristics. Cells within rays 2–6 are descended from the V6 lineage (Fig. 1A). The Hox gene product EGL-5 is expressed in the ray sublineages and differentiating cells of rays 3–6 but not in ray 2 or in the rays descended from the V5 (ray 1) or T (rays 7–9) lineages (Ferreira et al., 1999) (Figs. 1A, J). Several features of the *egl-5* null mutant phenotype suggest that in the absence of *egl-5*, rays 3–5 adopt a ray 2 identity. First, in *egl-5* null mutants [*egl-5(0)*], rays 3–5 assume the morphological identity of ray 2, clustering together at the ray 2 position and fusing with this ray (ray 6 is not generated, see Fig. 3F) (Chisholm, 1991; Chow and Emmons, 1994). Second, R5A no longer expresses dopaminergic fate and R3A to R5A can no longer be induced to express dopaminergic fate ectopically when exposed to exogenous DBL-1 (TGF β) ligand. In these respects, they now resemble R2A (Lints and Emmons, 1999). Third, rays 3–5 express elevated levels of EPH-4, an ephrin necessary for ray morphogenesis, also a characteristic property of ray 2 (Hahn and Emmons, 2003).

To extend these observations to the RnB neurons, we examined the expression of 5HT and FaRPs in the *egl-5* null mutant *egl-5(u202)*. In *egl-5* mutant males, the RnB neurons of rays 3–5 no longer expressed their wild-type ray-specific neurotransmitter patterns and instead express an R2B neurotransmitter profile, namely, FLP-X and *flp-6::GFP* (compare Figs. 2A, 3A with Figs. 2B, 3B, respectively; Table 1; Fig. 2C). Thus, *egl-5* defines the differentiated properties of all three terminal ray cells in

rays 3–5. The posterior to anterior transformation of the subtype properties of R3B to R5B to those of R2B is consistent with the observed changes in rays 3–5 morphology and RnA neuron properties and reveals that *egl-5* promotes the identities of these rays over the identity of ray 2 (Fig. 3F).

TGF β signal DBL-1 is required for ray 5 identity

The V6 rays are further diversified by a TGF β -family signal. Analysis of the effects of this signaling pathway revealed that it too coordinately specified multiple ray properties and hence may be regarded as a ray identity-determining factor. A TGF β signaling pathway known as the *dbl-1* pathway after the ligand, DBL-1, patterns several structures in the male tail (Morita et al., 1999; Suzuki et al., 1999). We showed previously that among the V6 rays of wild-type animals, a DBL-1 signal stimulates the expression of dopamine by R5A (Lints and Emmons, 1999). Furthermore, ubiquitous expression of the ligand from a heat-shock-driven transgene revealed that all V6 RnA neurons, except R2A, are to varying degrees competent to respond in this way to DBL-1. In addition, the *dbl-1* pathway is necessary for correct specification of the morphogenetic identity of ray 5 (Krishna et al., 1999; Morita et al., 1999; Savage et al., 1996; Suzuki et al., 1999); hence, it presumptively affects the identity of R5st.

To determine whether the *dbl-1* pathway also patterns the RnB neurons of the V6 lineage, we examined expression of RnB-neuron-specific neurotransmitter genes in loss-of-function mutants for the *dbl-1* pathway. In mutants affecting several steps of the *dbl-1* pathway, 5HT was ectopically expressed in R5B but unaffected in the remaining V6 rays (Figs. 2D, 3C; Table 1). Thus, DBL-1 signaling represses 5HT expression in R5B. By contrast, DBL-1 signaling is necessary for *flp-5*, *flp-6* and *flp-17* expression in R5B. In pathway mutants, expression of *flp-5*, *flp-6* and *flp-17* was lost in R5B. Neurotransmitter marker expression in other V6 RnB ray neurons was unaffected by pathway inactivation indicating that DBL-1 signaling is specifically required for ray 5 identity (Figs. 2D, E, 3C; Table 1, data not shown). Temperature-shift experiments with a temperature-sensitive allele of the *dbl-1* pathway Type II receptor gene [*daf-4(m592ts)*] revealed that the temperature-sensitive period for patterning 5HT expression was during the ray sublineage (Figs. 4A, B, D, E). A similar result was obtained by timed DBL-1 induction from a heat shock transgene (Figs. 4C, F). This is the same interval as was shown previously for patterning ray morphology (Lints and Emmons, 1999; Savage et al., 1996; data not shown) and dopamine expression (Fig. 4; Lints and Emmons, 1999). Thus, the *dbl-1* pathway affects properties of all three branches of the ray 5 sublineage and may be considered to define an overall ray 5 identity. Its function is not required for the identities of the remaining V6 rays.

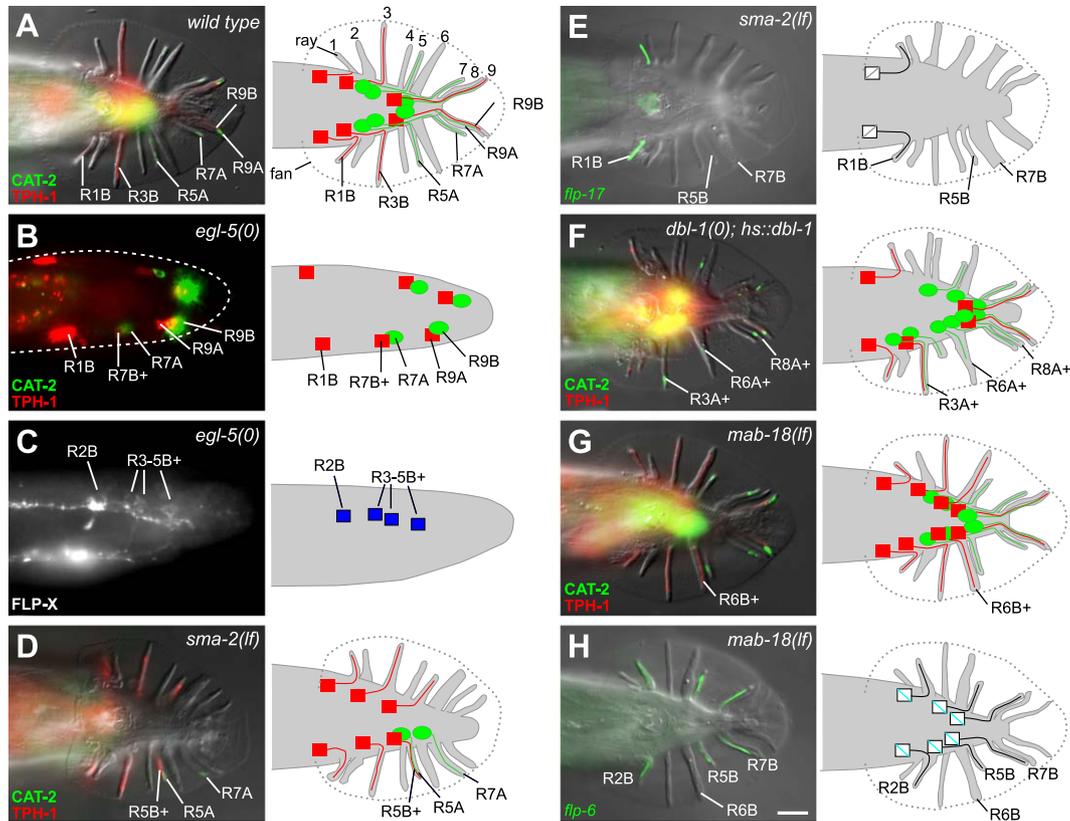


Fig. 2. *egl-5*, *mab-18* and *dbl-1* pathway activities are required for correct patterning of ray neurotransmitter phenotypes. Ray neurotransmitter fate expression patterns of wild-type and mutant adult males. DIC and fluorescent micrographs of the same animal superimposed (A, D–H) or fluorescent micrographs alone (B, C) are shown. Ventral view (A, B, D–H), lateral view (C); anterior left in all images. The dopaminergic and serotonergic fates were visualized using CAT-2::YFP (false-colored green; Lints and Emmons, 1999) and TPH-1::CFP (false-colored red; Sze et al., 2000), respectively. Yellow fluorescence corresponds to areas where YFP- and CFP-expressing cells overlap. + indicates ectopic fate expression. Cartoon version of each image is shown along side. The code for neurotransmitter fate and neuron type is as per Fig. 1J. Scale bar = 10 μ m. (A) Wild type. Dopaminergic fate marker CAT-2::YFP is expressed in neurons R5A, R7A and R9A and serotonergic fate marker TPH-1::CFP in R1B, R3B and R9B neurons. (B) *egl-5(u202)*. *egl-5* loss of function disrupts male tail retraction and so rays and fan do not extend from the body. Body outline is indicated by the grey dotted line. CAT-2::YFP and TPH-1::CFP are absent from the neurons of *egl-5*-dependent rays, R5A and R3B, respectively; TPH-1::CFP is ectopically expressed in R7B. (C) *egl-5(u202)* male stained with anti-FMRF-amide antisera, which visualizes FLP-X. FLP-X, is present in R2B and ectopically in R3B, R4B and R5B. (D) *sma-2(e502)*. Several ray morphological abnormalities characteristic of DBL-1 pathway mutants are apparent: on the uppermost side, rays 7 and 6 have fused and ray 9 has a ray 8 morphology; on the lowermost side, ray 7 has a ray 6 morphology and rays 8 and 9 have fused. CAT-2::YFP is present in R5A on the lowermost side and R7A on both sides. TPH-1::CFP is present in R1B and R3B and ectopically in R5B in both sides. Neither reporter is present in neurons of ray 9. (E) *sma-2(e502)* male expressing *flp-17::GFP* marker. Ectopic expression of 5HT in R5B (shown in D) is associated with complete loss of *flp-17::GFP* expression from this neuron and from R7B. *flp-17::GFP* expression in the ray 1 R1B neuron is unaffected. (F) CAT-2::YFP and TPH-1::CFP expression patterns after heat shock induced expression of a *hs::dbl-1* transgene. A *dbl-1(wk70)* adult male carrying a *hs::dbl-1/cat-2::YFP/tp-1::CFP* transgenic array (*bxEx70*, *bxEx71*) after heat-shock treatment at the Rn stage. *hs::dbl-1* gene activation on both sides restores rays 5, 7 and 9 morphology and their wild-type dopaminergic and serotonergic fate patterns (compare to D). On the lowermost side, it induces ectopic dopaminergic fate in ray neurons R3A, R6A and R8A and on the uppermost side suppresses serotonergic fate in ray 3 neurons, R3B. (G) *mab-18(bx23)*. On both sides, ray 6 has adopted a thin ray morphology and is fused with ray 4, displacing ray 5 to the posterior and TPH-1::CFP is ectopically expressed in R6B. (H) *mab-18(bx23)* expressing *flp-6::GFP* marker. Ectopic expression of 5HT in the ray 6 R6B neuron (shown in G) is associated with the loss of *flp-6::GFP*. *flp-6::GFP* expression in R2B, R5B and R7B neurons is unaffected.

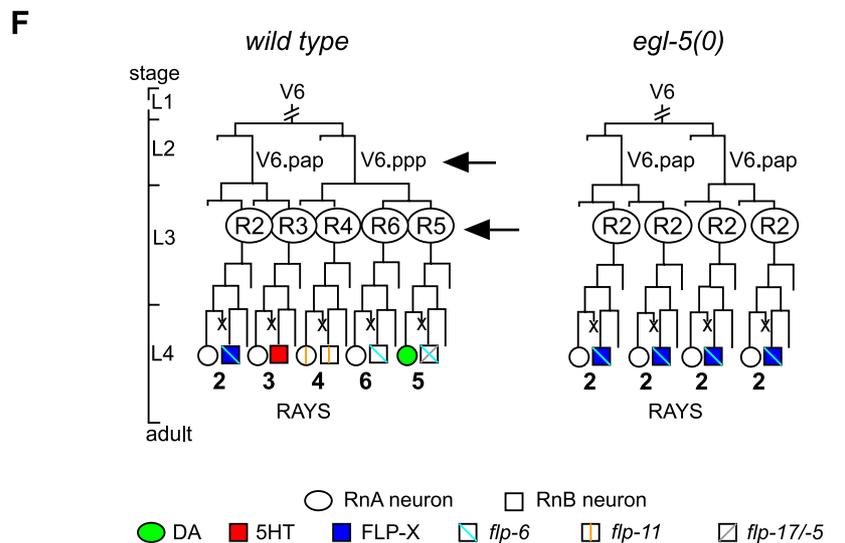
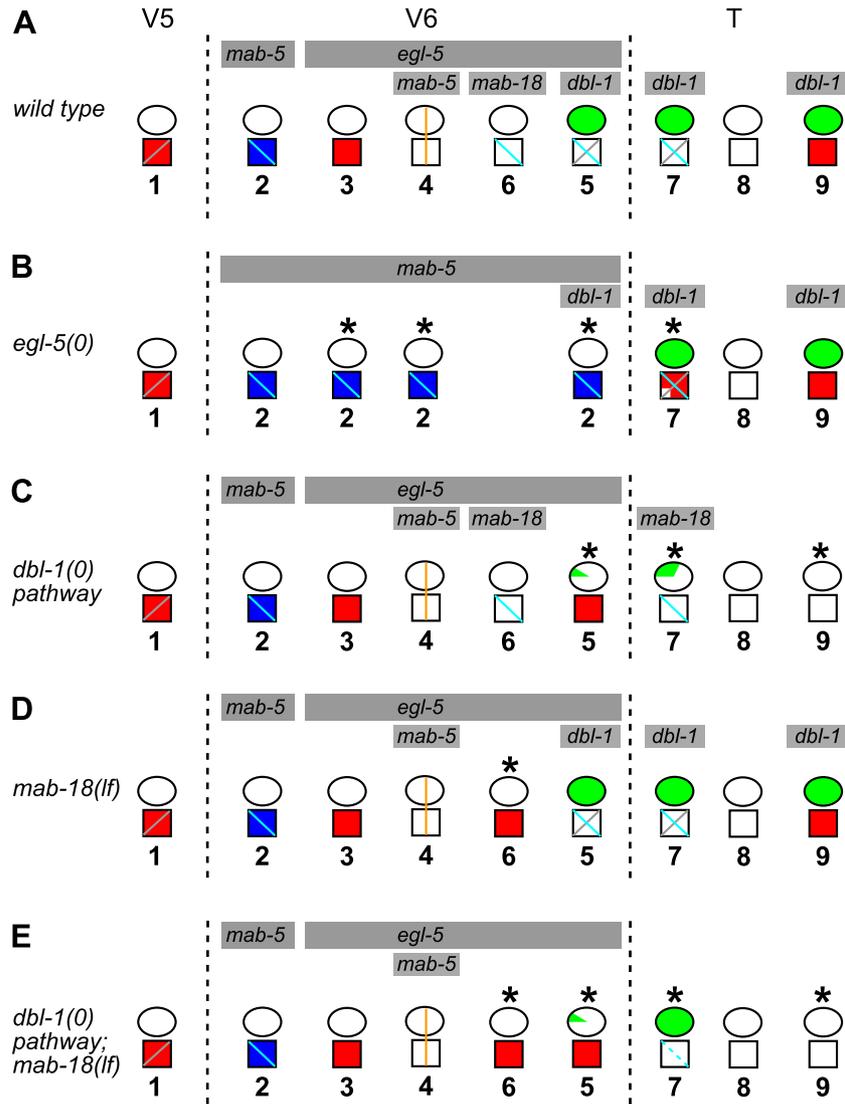
In the absence of *dbl-1* pathway activity, the neurotransmitter expression pattern in ray 5 is nearly identical to that in ray 3 (Fig. 3C; Table 1). R5B becomes serotonergic like R3B and expression of R5B-specific FaRPs *flp-5*, *flp-6* and *flp-17* is lost. The only difference from ray 3 is that approximately 10% of R5A neurons continue to express dopamine, whereas R3A neurons never express dopamine in this background. In a further similarity, we showed previously that *mab-23* represses dopamine expression in

both R3A and R5A in the absence of the DBL-1 signal (Lints and Emmons, 2002). Thus, with the exception of their infrequent expression of dopamine, ray 5 neurons resemble ray 3 neurons in the absence of *dbl-1* pathway activity.

Considering morphogenetic identity, in the absence of a DBL-1 pathway signal, ray 5 sublineage cells move ventrally to assume a position similar to those of the other V6 rays and ray 5 fuses with ray 4 in 10% of male tail

sides (Hahn and Emmons, 2003; Savage et al., 1996). Thus, in the absence of a DBL-1 pathway signal, R5st may take the morphogenetic identity of R4st so that affected

rays express a mixture of ray 3 and 4 properties. Even so, these data are consistent with the idea that loss of *dbl-1* pathway function causes ray 5 cells to adopt fates charac-



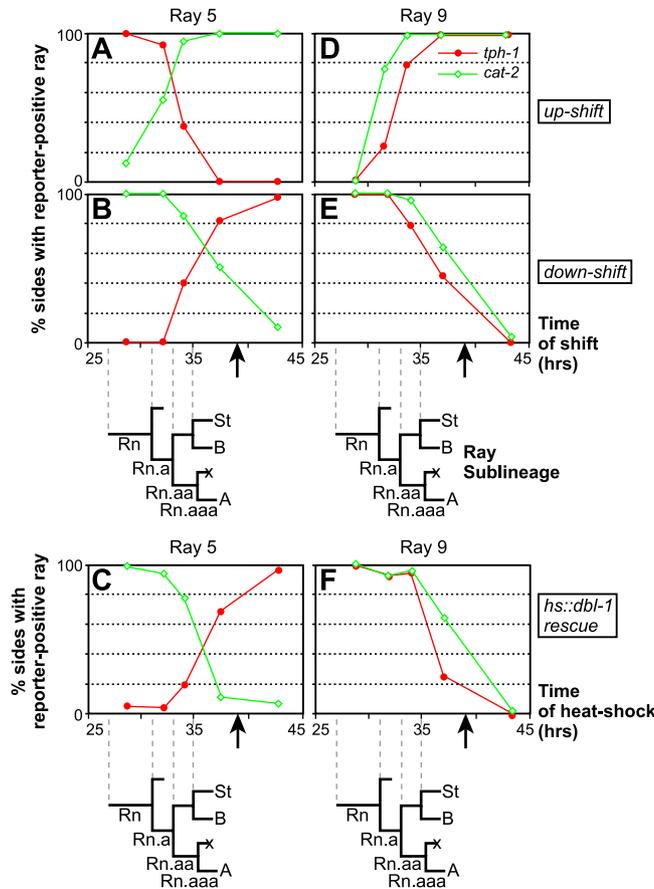


Fig. 4. The *dbl-1* pathway acts during the ray sublineage to define multiple ray identity characteristics. (A, B, D, E) Temperature shift. At the stage of the ray sublineage shown, *daf-4(592ts); bxIs16 (cat-2::YFP/tph-1::CFP)* males were shifted either from 15°C to 25°C (up-shift) or from 25°C to 15°C (down-shift), allowed to mature to adulthood, then scored for ray reporter expression and morphology. In selected cases, animals were stained for dopamine or serotonin (see Materials and methods). The number of sides scored for each stage (up-shift, down-shift) was as follows: Rn (28, 18); Rn.a (31, 48); Rn.aa (28, 31); Rn.aaa (ca. 38 h, terminal ray cells formed but undifferentiated) (24, 32); adult (ca. 44 h) (35, 118). Arrows indicate earliest time at which CAT-2::YFP and TPH-1::CFP can be detected in ray neurons. (C, F) Rescue of the *dbl-1(wk70)* dopaminergic/serotonergic patterning defect by *hs::dbl-1* gene induction at various stages. Animals carrying *bxEx71, bxEx72 (hs::dbl-1/cat-2::YFP/tph-1::CFP)* were heat-shocked at 30°C for 30 min at the stages shown. Number of sides scored for each stage was as follows: Rn (34); Rn.a (36); Rn.aa (23); Rn.aaa (ca. 38 h) (32); 41 h (10).

teristic of a more anterior *egl-5*-dependent ray identity. *egl-5* expression thus appears to define ray 3 identity for all three ray cells, and *egl-5* plus a DBL-1 signal defines ray 5 identity.

The DBL-1 signal specifies ray 5 neuron identity

In wild type, DBL-1 is expressed in several neurons in the male tail but the source relevant to patterning the rays

Fig. 3. Summary of neurotransmitter patterns in wild-type, *egl-5*, *mab-18* and the *dbl-1* pathway mutants. (A–E). Frequency and distribution of DA-, 5HT- and FMRF-amide-positive rays in wild type and mutant males. The percentage of male sides that showed expression of dopaminergic or serotonergic fate reporters (*cat-2* and *tph-1*, respectively) in a specific ray neuron is indicated by the proportion of the circle (RnA neurons) or square (RnB neurons) shaded. An asterisk indicates a change in ray identity. For *flp* reporters, neuronal expression was either 100% or 0% except in *mab-18* mutants (D) where 4% and 5% of male sides expressed *flp-11::GFP* and *flp-6::GFP* in ray 6, respectively, and in *dbl-1* pathway; *mab-18* double mutants (E) where 50% of male sides expressed *flp-6::GFP* in ray 7. In *dbl-1* pathway mutant backgrounds, the frequencies shown include fusions involving ray 5, 7 or 9 identities. In *mab-18* mutants, ray 6 identities include fusions with ray 4. In general, fused rays contain a single, reporter-positive neuron likely to correspond to the neuron that normally expresses the reporter in wild type (Lints and Emmons, 1999) or belongs to the ray that is positive when rays are unfused. The bars above indicate the predicted expression domains of the regulatory genes in wild type and mutant backgrounds. In *egl-5(0)* mutants, the MAB-5 domain of expression is predicted to expand to include rays 3–5, based on the observed transformation of these rays to a ray 2 (*mab-5*-dependent) identity and on interactions between MAB-5 and EGL-5 in other tissues where their domains of activity are juxtaposed (Salser et al., 1993). In *egl-5* mutants, MAB-18 is absent from ray 6 because this ray is not specified (F; Zhang, 1996). DBL-1 ligand production is predicted to be wild type in *egl-5* mutants, based on the observation that expression of DBL-1-dependent neurotransmitter fates in rays 7 and 9 are unaffected in this background. In *dbl-1* pathway mutants, ectopic expression of MAB-18 in ray cells likely to correspond to those of ray 7 is associated with transformation of ray 7 to a ray 6 morphology (Zhang, 1996). *n* = 30–150 sides scored per genotype per marker. (F) Predicted alteration in the V6 lineage caused by *egl-5* loss of function. Arrows indicate the two stages of the lineage where *egl-5* function is required. Corresponding larval stages are indicated by the scale to the left.

has not been determined (Suzuki et al., 1999). Its distribution appears to be nonuniform (Lints and Emmons, 1999). Heat-shock induction of DBL-1 revealed that, among the V6 rays, R3A-R6A are all to some degree competent to express dopamine upon DBL-1 induction (Lints and Emmons, 1999; Fig. 2F). To test further the relationship between rays 3 and 5 identities and to determine whether the DBL-1 signal is sufficient to specify ray 5 identity, we examined serotonin expression using a *tph-1* reporter in similar heat-shock induction experiments. A *dbl-1* null background was used to eliminate the patterned expression of the endogenous ligand. Upon heat-shock induction of DBL-1, 5HT expression was lost in R5B, thus restoring the wild-type non-serotonergic phenotype of this neuron as expected (Fig. 2F). A low frequency of 5HT induction was observed in R4B and R6B (Table 1; data not shown). Thus, as with dopamine expression in the RnA neurons, several of the V6 RnB neurons are competent to respond to the DBL-1 signal by expressing 5HT. Significantly, heat-shock induction of DBL-1 substantially repressed 5HT expression in R3B (Fig. 2F). DBL-1 signal is, therefore, sufficient to generate the difference between wild-type R3B and R5B. However, as is also observed for induction of dopamine expression in R3A, inhibition of serotonin is less complete in ray 3 than in ray 5. Therefore, cells of rays 3 and 5 may differ by factors that alter their sensitivity to the DBL-1 signal but not the specificity of their response. Alternatively, it is possible that, for unknown reasons, ray 3 cells and ray 5 cells are differentially exposed to the ubiquitously expressed ligand in our experiments.

The Pax-6 isoform MAB-18 is required for ray 6 identity

Ray 6 is unique among the rays in requiring the Pax-6-related transcription factor MAB-18 for its distinct tapering morphology and morphogenetic identity (Zhang and Emmons, 1995). We therefore tested whether *mab-18* was a ray identity gene that accounted for the subtype properties of the ray 6 neurons as well. We showed previously that in *mab-18* mutants, the ray 6 structural cell appeared to undergo a specific transformation to a ray 4 morphogenetic identity and ray 6 fused with ray 4 (Zhang and Emmons, 1995). MAB-18 is expressed in several rays in each branch of the ray sublineage but is cytoplasmic until the cells begin to differentiate. At that time, MAB-18 expression becomes nuclear only in the three cells of ray 6 and is lost from the other rays. Its activity is therefore likely to be cell-autonomous within ray 6 cells (Zhang et al., 1998).

To determine whether *mab-18* specified the subtype properties of the ray 6 neurons, we analyzed neurotransmitter expression in the loss-of-function mutant *mab-18(bx23)*. In this background, R6B no longer expressed *flp-6* and instead expressed 5HT and occasionally the ray 4

neuron marker *flp-11* (Figs. 2G, H, 3D; Table 1). Expression of neurotransmitters by the other rays was unaffected. Thus, as for ray 5 in *dbl-1* pathway mutants, loss of *mab-18* function from ray 6 causes R6B neurons to adopt the neurotransmitter identity of the most anterior *egl-5*-dependent ray, ray 3. In a further similarity, not all ray 6 cells are uniformly affected in *mab-18* mutants and transformed rays express a mixture of ray 3 and 4 characteristics, ray 4 morphogenetic identity and occasionally ray 4 neuron fate. Overall, however, loss of *mab-18* function results in transformation of ray 6 to a more anterior *egl-5*-dependent ray identity.

To further examine the properties of R6B, we determined the effect on ray 6 of ubiquitous DBL-1 expression from the heat-shock promoter in a *mab-18* mutant background. If both ray 6 neurons had ray 3 identities, heat-shock induction was expected to repress 5HT expression in the R6B neuron and activate dopamine expression in the R6A neuron. However, expression of markers for these neurotransmitters was unaffected by heat shock (Table 1). Thus, in a *mab-18* mutant background, R6A and R6B are not respectively equivalent to R3A and R3B. This observation indicates that R6A and R6B differ from R3A or R3B by one or more additional factors besides MAB-18. Unlike the structural cell, which may resemble R4st, the properties of R6A and R6B in the absence of MAB-18 are not identifiable with those of any other ray. Hence, ray 6 identity is specified by multiple factors, or ray 6 lacks factors present in the other rays.

Patterning the T rays

The selector genes, which specify differences between rays 7 and 9 from the T lineage, are unknown. Unlike the V5 and V6 lineages, expression of rays in the T lineage requires the gene *mab-19* (Sutherland and Emmons, 1994). Thus, the genetic program underlying ray development in the T lineage is distinct, although the outcome, such as, for example, the pattern of neurotransmitter expression in rays 5 and 7, may be similar. Our examination of neurotransmitter expression patterns in various mutant backgrounds allowed us to gain further insight into the patterning of ray identities in the T lineage.

Analysis of neurotransmitter expression in an *egl-5* mutant background uncovered an unexpected cell-non-autonomous effect on the T lineage. Among the rays descended from V5 and T, the neurotransmitter expression pattern was generally the same in an *egl-5* mutant background as in wild type. However, there was one exception. In ray 7, R7B ectopically expressed 5HT at high frequency in *egl-5(u202)* (Figs. 2B, 3B, Table 1). This effect was limited to 5HT expression; expression of the other ray 7 neurotransmitters, namely, *flp-5*, *flp-6* and *flp-17* by R7B and dopamine by R7A, was unaffected. Since EGL-5 is not expressed in ray 7 cells (Ferreira et al., 1999), this is a cell-non-autonomous effect. *egl-5* activity

may result in generation of a signal that inhibits 5HT expression by R7B, hence helping to pattern the T lineage. Alternatively, *egl-5* may inhibit generation of a signal that stimulates 5HT expression by R7B. In this case, *egl-5* acts to prevent any non-autonomous effect in wild type. The source and nature of the signal is unknown; it could arise from cells of rays 2–5 or from any one of several EGL-5-expressing non-ray cells present in the tail (Ferreira et al., 1999).

The *dbl-1* pathway helps to pattern T rays as it does for V6 rays. In *dbl-1* pathway mutants, ray 7 frequently has the tapered morphology of ray 6 and fuses with ray 6 (Savage et al., 1996). In addition, expression of dopamine by R7A and R9A is stimulated by the *dbl-1* pathway (Lints and Emmons, 1999). We examined 5HT and neuropeptide expression and found that the *dbl-1* pathway was also required for expression of 5HT by R9B and *flp-5* and *flp-17* by R7B (Figs. 2D, E; data not shown; Fig. 3C; Table 1). Thus, as with V6 rays, the *dbl-1* pathway specifies properties of all three ray cells in the T lineage.

With respect to neurotransmitter expression pattern, ray 7 resembles ray 5. Both of these rays open on the dorsal surface of the fan; hence, their similarity extends to their morphology as well. In ray 5, these properties require *egl-5*, yet ray 7 does not express this Hox gene, and with the exception noted above, its properties are not affected by *egl-5* mutations. This indicates that a similar spectrum of differentiation genes is directly or indirectly activated by a different set of transcription factors in ray 7.

The difference between rays 5 and 7 was further emphasized by their differing response to absence of the DBL-1 signal. In ray 5, this resulted in ectopic expression of 5HT, but in ray 7 it did not (Figs. 2D, 3C, Table 1). Further, in ray 7, as in ray 5, dopamine expression by R7A and *flp-5* and *flp-17* expression in R7B were lost, but *flp-6* expression in R7B was retained (Fig. 2E; Table 1; data not shown). Since in *dbl-1* pathway mutants, ray 7 appeared to assume the morphogenetic identity of ray 6 and sometimes fused with ray 6, the retention of the R6B neurotransmitter *flp-6* suggested that ray 7 assumes an overall ray 6 identity. This suggested that MAB-18 might be ectopically expressed in ray 7 in a *dbl-1* pathway mutant background. Indeed, we previously gained evidence of such ectopic expression (Zhang, 1996). We therefore examined neurotransmitter expression in ray 7 in *dbl-1* pathway; *mab-18* double mutants. As predicted, expression of *flp-6* was partially lost (Fig. 3E; Table 1; data not shown). Surprisingly, however, dopamine expression was restored in R7A (Table 1; data not shown). Evidently, in the context of unknown factors specific to R7A, *mab-18* inhibits *cat-2* expression when the *dbl-1* pathway is inactive. Ectopic expression of MAB-18 in *dbl-1* pathway mutants suggests that MAB-18 is acting cell-autonomously. It remains possible, however, that this could be a cell-non-autonomous effect emanating from a MAB-18-expressing cell (e.g., ray 6).

Discussion

A complex pattern of neurotransmitter expression among the rays

Earlier studies established that certain subsets of the rays expressed the monoamine neurotransmitters dopamine and serotonin (Lints and Emmons, 1999; Loer and Kenyon, 1993; Sulston et al., 1980). Here, we show that ray neurons also express FMRFamide-related neuropeptides in complex patterns. The functional reason for this complexity is unknown, but coincides with our observations that the RnA and RnB neurons from different rays have different post-synaptic targets in the preanal ganglion (S. W. E., D. Albertson, D. H. Hall, and H. Eckholdt, unpublished data). Although details of the circuitry in the male tail and the functions of individual ray neurons within it are unknown, the complexity of the rays as a group suggests that the fan and rays comprise a compound sensory organ that provides multifactorial input about the position of the tail on the hermaphrodite body. Specification of individual neuronal subtype properties together with ray position provides information about where a particular stimulus is received to guide the male's copulatory behavior.

Hox genes are ray identity selector genes

As originally defined by Garcia-Bellido (1975), selector genes have several properties. Selector genes select between alternate developmental states, acting cell-autonomously, instructively and combinatorially. Their function is confined to cell lineage-restricted compartments, within which they endow the cells not only with their region-specific characteristics but also with specific cell recognition and cell adhesion properties that keep them physically together. For example, in vertebrates, members of the *Hox* paralog groups 1–4 (*Hox-1* to *Hox-4* genes) function as selector genes and play a critical role in patterning rhombomeres of the hindbrain. Selective deletion of Hox genes results in loss or partial transformation of individual rhombomeres, manifested as fusion of segments and changes in neuron populations (Rossel and Capecchi, 1999; Studer et al., 1998). In the *C. elegans* male tail, the Hox genes *mab-5* and *egl-5* define ray identities and fit the functional definition of selector genes to a remarkable degree. Both genes are expressed in specific branches of the cell lineages leading to the rays, where several experiments have indicated that they act cell autonomously (Chisholm, 1991; Chow and Emmons, 1994; Ferreira et al., 1999; Kenyon, 1986; Salser and Kenyon, 1996). Based on observations of ray morphology and position, both *mab-5* and *egl-5* are necessary for posterior ray morphological identities; a loss of these gene products results in posterior-to-anterior transformations (Chisholm, 1991; Chow and Emmons, 1994). Evidence for an instructive role for *mab-5* was indicated by anterior-to-posterior morphological transformations in a gain-of-

function allele and in *mab-5* overexpression animals (Chow and Emmons, 1994; Salser and Kenyon, 1996). Similarly, the instructive role of *egl-5* is suggested by anterior-to-posterior ray identity transformations occurring in mutant backgrounds where *egl-5* is ectopically expressed in anterior lineage branches (Zhang and Emmons, 2001; Zhang et al., 2003). The roles of both genes in defining cell recognition and adhesion functions are implied by the frequent coalescence of the cells from two or more rays into a single fused ray when correct specification of ray identity is lost (Baird et al., 1991; Chisholm, 1991; Chow et al., 1995; Hahn and Emmons, 2003). Thus, here, as elsewhere, one function of the selector genes is to prevent cells from inappropriate commingling. The effectors of this property under Hox gene control have not yet been identified (Hahn and Emmons, 2003).

Ray 2 is a V6 lineage ground state

Selector genes act by altering the ground or default state of a cell. In *Drosophila melanogaster* antennae, legs, genitalia and analia make up a serially homologous set of ventral appendages that depend on different selector genes for their unique identities, namely, homeobox gene *homothorax* (*hth*) and Hox genes *Antennapedia* (*Antp*) and *Sex combs reduced* (*Scr*). Systematic elimination of these genes identified a leg-like structure as the ground-state ventral appendage (Casares and Mann, 2001). These general principles also apply to sensory element specification within a compound sensory organ. By analyzing multiple properties of the rays, we propose that *egl-5* acts as a selector gene for V6 ray identity. *egl-5* affects the properties of all three cell types of the ray sublineage. It is expressed in all but the most anterior of the V6 lineage branches and it alters the properties of all rays in which it is expressed, namely, rays 3–6. Ray 2, the most anterior V6 ray, normally does not express EGL-5, and in an *egl-5* mutant, all the V6 rays take the characteristics of ray 2. Therefore, ray 2 represents the *egl-5*(–) ground state. Interestingly, an anterior ground-state identity has also been identified for the rhombomeres of the zebrafish hindbrain. Deletion of the *pbx* genes, which encode essential DNA-binding Hox cofactors, results in loss of segmentation and transformation of rhombomeres r2–r6 to anterior r1, a non-Hox gene-expressing rhombomere (Waskiewicz et al., 2002).

Since ray 2 expresses *mab-5*, we cannot yet deduce the ground state for the ray sublineage in the absence of any selector gene activity. *mab-5* is not expressed in ray 1, and ray 1 is transformed to ray 2 if *mab-5* is expressed there (Chow and Emmons, 1994; Salser and Kenyon, 1996). Thus, ray 1 might represent a ray sublineage ground state. However, ray 1 might also express a different, unidentified selector gene. Similarly, we do not know what selector genes may participate in patterning the three rays, 7–9, derived from blast cell T. It seems most likely that the ray sublineage program itself specifies fully differentiated neu-

rons expressing neurotransmitters, receptors, and so forth. What this default set of properties is, representing the ground state for the ray sublineage itself, remains unknown. Its nature is of interest because it may be related to the primordial sublineage program that gave rise to the rays during evolution.

A combinatorial code for V6 ray identity

From our results, a partial picture of the combinatorial transcription factor code that specifies ray identity in the V6 lineage can be constructed (Fig. 5A). Expression of *egl-5* discriminates ray 3 identity from ray 2 identity. A DBL-1 signal discriminates ray 5 identity from ray 3 identity. This model, however, only partly explains the data and indicates where additional factors are required or where further assumptions must be made. For example, transformation of V6 rays uniformly to a ray 2 identity in *egl-5* mutants suggests that *egl-5* represses *mab-5* in most of the posterior lineage branches and predicts that *mab-5* comes on in these lineages in an *egl-5* mutant (Fig. 3B). In this event, ray 4 must contain a factor that prevents this antagonistic relationship, allowing both Hox genes to be expressed in the same cell. Ray 6 requires a factor to explain why in a *mab-18* background its morphology is that of ray 4 and its neurons have characteristics that partially overlap with ray 3. Ray 5 requires a factor to explain why its neurons are less dependent on and more sensitive to the DBL-1 signal than those of ray 3. Thus, the model is useful in pointing the directions for future studies.

The combinatorial codes underlying V6 ray identities include not only factors that coordinately pattern all three cell types within a ray but also regulators that define the characteristics of only one or two ray cells. One such regulator is DM-domain transcription factor gene *mab-23*. *mab-23* acts in V rays to control expression of neurotransmitter in one cell type, the RnA neuron (Fig. 5A). Expression of *mab-23* in these lineages is established independently of *mab-5*, *egl-5*, *lin-32* or the *dbl-1* pathway (R.L. and S.W.E, unpublished data). As discussed above, *egl-5* activity in the V6 rays confers a competence to adopt dopaminergic fate in RnA neurons (Lints and Emmons, 1999). *mab-23* suppresses this potential, blocking inappropriate expression of dopaminergic fate. However, *mab-23* activity is blocked in ray 5 by DBL-1 signaling and this allows the wild-type dopaminergic fate of R5A to be established (Lints and Emmons, 2002). Thus, *mab-23* contributes to the generation of ray diversity by altering, cell specifically, properties encoded by the more broadly acting selector genes. Ray-specific modulation of *mab-23* by DBL-1 suggests how new ray identities could be generated by altering the activity of such cell-type-specific factors in select ray lineages.

Combinatorial codes are also used to determine neuropeptide identity in other organisms, and, in particular, for the expression of FaRPs (Allan et al., 2003; Marqués et al.,

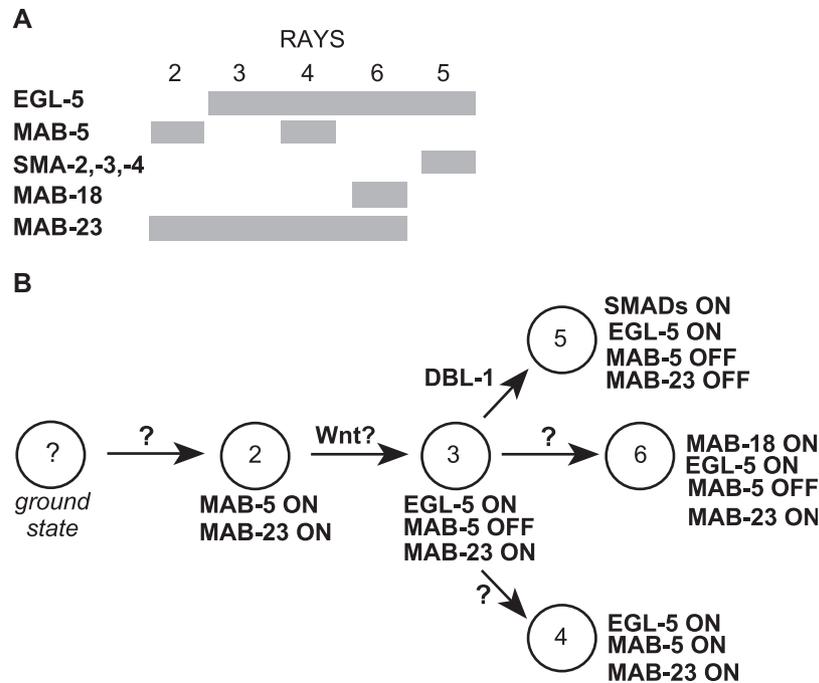


Fig. 5. Generation of V6 ray identities from a ground state. (A) Schematic showing combinatorial transcription factor codes for V6 ray identities. Each ray identity is defined by a distinct combination of transcription factors (left column). The grey bars indicate their domains of activity. SMA-2, -3 and -4 correspond to the downstream SMAD transcription factors of the DBL-1 pathway. DM-domain transcription factor MAB-23 is expressed in the RnA neurons of rays 1–4 and ray 6. (B) A schematic diagram showing transitions between transcriptional states. The numbers in circles are meant to represent the identity of the corresponding ray. The transcription factors listed are critical for defining these identities. Each state depicted potentially differs from adjacent states in the diagram by a single event. The arrows represent the cell extrinsic events in the developmental program that trigger, in a stepwise fashion, the activation of the critical transcription factor genes shown (see text).

2003). FaRP expression in the neuroendocrine Tv neurons in *Drosophila* is dependent on TGF β signals sent from the Tv target cells, the neurohemal organs. This signal is interpreted to determine FaRP expression only when the transcription factors Squeeze and Apterous are present; absence of either Squeeze or Apterous in the Tv neurons results in the lack of FaRP expression (Allan et al., 2003; Benveniste et al., 1998). Similarly, a TGF β signal acts in conjunction with specific transcription factors to establish *flp* gene expression in specific rays in *C. elegans*.

Establishment of the transcriptional states of the rays

The relationships between the transcriptional states of the V6 rays shown in Fig. 5A are represented schematically in Fig. 5B. In the figure, the developmental events that cause the transitions between states are represented by arrows. Developmental programs assign different fates to cells through the influence of asymmetrical, outside signals. Such signals may act on a cell after it is born, on cells as they divide to generate daughter cells with different fates, or at an earlier developmental time to establish a cell polarity and resulting lineage asymmetry that is propagated cell-autonomously through multiple cell generations (Horvitz and Herskowitz, 1992; Lin et al., 1998). We can identify or suggest some of the extrinsic influences that determine the asymmetric cell fates in the ray lineages. The first of these

establishes a difference between V6.ppp and V6.pap during the L2 larval stage, resulting in expression of *egl-5* in the progeny of the former (Fig. 3F). V6.ppp and V6.pap lie next to each other in the row of blast cells known as the seam, and both contain the necessary *egl-5* activator *mab-5* (Ferreira et al., 1999; Salser and Kenyon, 1996). However, *egl-5* transcription is initiated only in V6.ppp. We suggest that the difference between V6.ppp and V6.pap may be established by the Wnt pathway, which acts ubiquitously to establish a/p polarity in the *C. elegans* cell lineage (Lin et al., 1998). Mutations in various components of the Wnt pathway result in extensive abnormalities in the seam lineages (Herman et al., 1995; Sawa et al., 1996). A second event, also possibly due to the Wnt pathway, causes *egl-5* to be expressed in V6.pppp (R3), resulting in the difference between rays 3 and 2 (Fig. 3F). A third event is the reception by cells of ray 5 of a DBL-1 signal. What events cause the differences of rays 4 and 6 or that establish MAB-23 expression in RnA neurons are still unknown.

We have shown that even in an animal composed of a small number of cells, where cell fates are specified on a cell-by-cell basis during the cell lineage, anteroposterior patterning of serially homologous sensory structures is by a Hox selector gene mechanism. Our results are similar to results obtained for the patterning of motor neuron cell lineages and neurotransmitter expression along the *C. elegans* ventral cord (Kenyon, 1986; Salser et al., 1993). We

have extended these observations to the neuron subtype properties of sensory neurons. In the rays, it should be possible to identify the complete deterministic mechanism that leads to the complex neural pattern within a compound sensory organ.

Acknowledgments

We wish to thank J. Sze for the *tpH-1::GFP* reporter strain and plasmid; C. Savage-Dunn for *sma-2*, *-3*, *-4* double and triple mutant strains; M. Yandell and W. B. Wood for the *hs::dbl-1* plasmid; M. Barr for independent confirmation of the *flp* reporter ray expression patterns in wild type; and T. Lints and members of the laboratory for helpful discussions. Some nematode strains were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources. R. L. is a NARSAD Young Investigator. This work was supported by NIH Grant R01 NS30986 to S.W.E. and NSF Grants IBN-9808861 and IBN-9973581 and NIH Grants K02AG00708 and NS42459 to C.L. S.W.E. is the Siegfried Ullmann Professor of Molecular Genetics.

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