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Patterning of dopaminergic neurotransmitter identity among *Caenorhabditis elegans* ray sensory neurons by a TGFβ family signaling pathway and a *Hox* gene

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SUMMARY

We have investigated the mechanism that patterns dopamine expression among *Caenorhabditis elegans* male ray sensory neurons. Dopamine is expressed by the A-type sensory neurons in three out of the nine pairs of rays. We used expression of a tyrosine hydroxylase reporter transgene as well as direct assays for dopamine to study the genetic requirements for adoption of the dopaminergic cell fate. In loss-of-function mutants affecting a TGFβ family signaling pathway, the DBL-1 pathway, dopaminergic identity is adopted irregularly by a wider subset of the rays. Ectopic expression of the pathway ligand, DBL-1, from a heat-shock-driven transgene results in adoption of dopaminergic identity by rays 3-9; rays 1 and 2 are refractory. The rays are therefore prepatterned with respect to their competence to be induced by a DBL-1 pathway signal. Temperature-shift experiments with a temperature-sensitive type II receptor mutant, as well as heat-shock induction experiments, show that the DBL-1 pathway acts during an interval that extends from two to one cell generation before ray neurons are born and begin to differentiate. In a mutant of the *AbdominalB* class *Hox* gene *egl-5*, rays that normally express EGL-5 do not adopt dopaminergic fate and cannot be induced to express DA when DBL-1 is provided by a heat-shock-driven *dbl-1* transgene. Therefore, *egl-5* is required for making a subset of rays capable of adopting dopaminergic identity, while the function of the DBL-1 pathway signal is to pattern the realization of this capability.

Key words: TGFβ, dpp, BMP, Hox gene, Abdominal-B, Dopamine, Neurotransmitter, Neuron identity, *Caenorhabditis elegans*

INTRODUCTION

Specification of cell fate is key to understanding the development and function of a nervous system. In *C. elegans*, where cell lineages are reproducible and gene expression programs can be studied with single-cell resolution, it is possible to explore the progression of cell-state changes that lead to the generation of cells with individual identities. Here we describe studies taking advantage of the neurons of male sensory rays to address how neurons are programmed to adopt a particular neurotransmitter identity.

There are nine similar bilateral pairs of rays extending out of the body on each side of the male tail. Each ray comprises two sensory neurons (denoted A-type and B-type) and a support cell surrounded by a hypodermal sheath. Each ray consists of similar cells and is generated by repetition of a stereotyped cell sublineage. Yet each ray also has individual characteristics. These include position within the genital specialization, morphology, neurotransmitter usage, expression of a sensory receptor, transcription factor expression profile and functional role in mating behavior (Sulston and Horvitz, 1977; Sulston et al., 1980; Loer and Kenyon, 1993; Liu and Sternberg, 1995; Troemel et al., 1995; Salser and Kenyon, 1996; Zhang et al., 1998; Ferreira et al., 1999). Further individual characteristics may encompass expression of other sensory receptors and modalities, axon pathfinding and synaptic targets. Thus we may distinguish two developmental processes involved in generation of each ray: one is a program of neurogenesis that results in the generation of three differentiated cell types and the other is a pattern-formation process that assigns the characteristics that differ among the rays. The existence of two processes is supported by the identification both of genes required in all the rays and genes involved in specifying ray-specific properties (reviewed in Emmons, 1999).

Dopamine (DA) is expressed by R5A, R7A and R9A, the A-type sensory neurons present respectively in rays 5, 7 and 9 (Sulston and Horvitz, 1977; Sulston et al., 1980). Here we identify a TGFβ-family signaling pathway and a *Hox* gene that are involved in directing the dopaminergic fate specifically to these three neurons. *C. elegans* genes encoding components of TGFβ-like signaling cascades have been defined by genome sequence analysis and by genetic studies (reviewed in Chervitz et al., 1998; Ruvkun and Hobert, 1998; Padgett, 1999). A TGFβ pathway involved in defining male ray morphology, which we

will refer to as the DBL-1 pathway, has been defined by mutations in six genes. The pathway ligand, encoded by a gene variously named *dbl-1* and *cet-1*, is a member of the Vg1/Dpp/BMP subfamily of TGFβ molecules, most closely resembling Nodal (Morita et al., 1999; Newfeld et al., 1999; Suzuki et al., 1999). *sma-6* and *daf-4* encode type I and type II receptors, respectively (Estevez et al., 1993; Krishna et al., 1999). *sma-2, sma-3* and *sma-4* encode SMAD proteins likely to act as downstream transducers (Savage et al., 1996). The DAF-4 receptor and the signal transduction pathway have been shown to act cell autonomously in specification of ray morphology (Savage et al., 1996). *dbl-1* is expressed in several neurons within the tail, but the source important for ray morphology has not been identified (Suzuki et al., 1999).

The *AbdominalB* class *Hox* gene *egl-5* is one of two *Hox* genes known to function in ray development. The *Antennapedia* homolog *mab-5* is required for generation of rays 1-6, whereas *egl-5* is required for generation of ray 6 (Kenyon, 1986; Chisholm, 1991). Both genes also function in patterning ray morphological identities (Chisholm, 1991; Chow and Emmons, 1994; Salser and Kenyon, 1996). *mab-5* and *egl-5* act cell-autonomously and are expressed in complex patterns in lineages giving rise to subsets of rays (Kenyon, 1986; Chisholm, 1991; Salser and Kenyon, 1996; Ferreira et al., 1999).

We find that the DBL-1 pathway is required for patterning the dopaminergic phenotype and that *egl-5* is necessary for expression of DA by a subset of the rays. Our results illustrate how two characteristics of sensory structures, morphology and neurotransmitter usage, may be coordinated by utilizing common regulatory components. They also show how a robust wild-type cellular pattern results from the combined actions of two independent patterning systems.

MATERIALS AND METHODS

C. elegans strains and cultures

Nematodes were grown and maintained as described in Brenner (1974). Bristol (N2) and *him-5(e1490)V*, which generates males at high frequency, were used as reference wild-type strains. Unless otherwise stated, strains were maintained at 20-22°C. The following mutations were used: LG I: *daf-8(m85)*; LG II: *cat-2(e1112), sma-6(wk7)* and *(wk8);* LG III *egl-5(u202), daf-4(m592ts), sma-2(e172)* and *(e502), sma-3(e491)* and *(e637), sma-4(e729), pha-1(e2123ts)*, LGIV: *him-8(e1489), daf-1(m40), unc-129(ev557);* LGV: *dbl-1(ev580)* and *(wk70)*; LG X: *daf-3(mgDf90)*. Except for *daf-4(m592ts)*, all mutant strains have been described previously (Brenner, 1974; Sulston et al., 1975; Georgi et al., 1990; Chisholm, 1991; Wang et al., 1993; Granato et al., 1994; Patterson et al., 1997; Colavita and Culotti, 1998; Krishna et al., 1999; Suzuki et al., 1999; consult also Riddle and Albert, 1997). *daf-4(m592ts)* is a temperature-sensitive missense mutation: animals are Daf at 25°C, nonDaf but Sma at 20°C and wild type at 15°C (D. Riddle, personal communication).

Mutant strains carrying transgenic constructs were generated by appropriate crosses between the mutant strain and transgenic lines. The following duplicate pairs (independently generated) of unintegrated transgenic arrays all contained wild-type genomic DNA: *bxEx35* and *bxEx36*, consisting of *cat-2::gfp* reporter EM#282 and selectable marker pRF4(*rol-6(su1006)*); *bxEx44* and *bxEx45*, consisting of *cat-2::gfp* reporter EM#282, the empty heat-shock vector pPD49.78 and selectable marker pBX-1(*pha-1(+)*); *bxEx46* and *bxEx47*, consisting of the *cat-2::gfp* reporter EM#282, the heat-shock *dbl-1* plasmid pMYHSdbl-1, and selectable marker pBX-1(*pha-1(+)*). The integrated transgenic array *mnIs17* consisted of *osm-6::gfp* and selectable marker *unc-36(+)* (gift of C. Spike and R. Herman).

Formaldehyde-induced fluorescence

Whole animals were stained for dopamine by means of a formaldehyde-induced fluorescence (FIF) technique (Sulston et al., 1975; Jagdale and Gordon, 1994) as modified by Sawin (1996). One to five animals were placed on a microscope slide in a 5 μl drop of paraformaldehyde solution (4% paraformaldehyde in 0.1 M Na_2HPO_4/KH_2PO_4 buffer, pH7.2) and incubated at room temperature for 5 minutes. Excess liquid was wicked off with a strip of 3 MM paper and the slide was heated for 10 minutes on an aluminium block at 96-100°C, and then cooled to room temperature. A drop of glycerol and a coverslip were placed on top of the treated worms. Fluorescence was observed at ×1000 with a Zeiss 487905 filter set (excitation, 395-440 nm; emission, 470 nm long pass).

cat-2 cloning, rescue and *gfp* reporter constructs

Predicted gene B0432.5 was identified as a candidate tyrosine hydroxylase gene by BLAST search with rat and *Drosophila* TH genes as query sequences (Grima et al., 1985; Neckameyer and Quinn, 1989). B0432.5 was isolated on a 9.3 kb *Eco*RV fragment from cosmid C09D7. This *Eco*RV fragment was inserted into pSK(+) (Stratagene) at the *Sac*I site, which was cleaved by *Ecl*136 to generate blunt ends, generating plasmid EM#287. Microinjection of EM#287 (10 ng/μl) together with pRF4 (which contains the dominant allele of *rol-6, su1006*, Mello et al., 1991) (100 ng/μl) into *cat-2(e1112)* hermaphrodites resulted in FIF in 95% of transformants (identified by roller phenotype), demonstrating rescue of the *cat-2(e1112)* mutation. Five independently generated transgenic lines gave similar results. Five independent control lines, generated by microinjection of *cat-2(e1112)* animals with pRF4 DNA alone, had no FIF.

To identify the *e1112* mutation, B0432.5 was amplified by PCR from *cat-2(e1112)* genomic DNA, cloned and sequenced on both strands. Sequences obtained from two independently amplified clones contained a C→T mutation that is predicted to result in a termination codon at position 161 in the predicted TH protein. Sequences obtained from DNA amplified from *cat-2(e1112)* were compared to sequences obtained from control DNA amplified both from *him-5 (e1490)* and N2 strains.

cat-2::gfp reporter construct EM#282 was constructed by in-frame insertion of a 2.6 kb *Hin*dIII–*Xba*I genomic fragment from C09D7 into the *Hin*dIII and *Nhe*I sites of *gfp* vector pPD95.75 (courtesy of A. Fire). This fragment contains 1.5 kb upstream genomic sequence and DNA encoding 245 amino acids of the predicted TH protein. The fusion protein is predicted to be non-functional because it lacks most of the catalytic region (Goodwill et al., 1997).

Generation of transgenic animals

Transgenic nematodes were generated by microinjection of DNA following Mello et al. (1991). All arrays containing the *cat-2::gfp* reporter, EM#282, were generated by microinjection of linearized blunt-ended fragments. Transgenic lines carrying extrachromosomal transgenic arrays *bxEx35* and *bxEx36* (Figs 2, 3, 4 and 7) were generated by co-injection of EM#282 (1 ng/μl), pRF4 (*rol-6(su1006)*) (1 ng/μl), and *C. elegans* genomic DNA (75 ng/μl) into strain *him-5(e1490)*. To examine the affects of DBL-1 expression from a heat-shock-driven transgene, transgenic lines carrying extrachromosomal arrays *bxEx46* or *bxEx47* (Figs 2, 5, 6 and 7) were generated by coinjection of EM#282 (1 ng/μl), pMYHSdbl-1 (1 ng/μl), pBX-1 (1 ng/μl) and *C. elegans* genomic DNA (75 ng/μl) into *pha-1(e2123ts);him-5(e1490)*. pMYHSdbl-1 (kindly provided by M. Yandell and W. B. Wood) contains a *dbl-1* cDNA placed under control of the heat-shock promoter of vector pPD49.78. pBX-1 contains a wild-type copy of the *pha-1* gene (Granato et al., 1994). Control arrays *bxEx44* and *bxEx45* were constructed similarly with the heat-shock vector pPD49.78 (A. Fire) instead of pMYHSdbl-1. Transgenic *pha-1*

lines were maintained at 25°C. The *osm-6* array used, *mnIs17*, is an integrated derivative of *mnEx64* (Collet et al., 1998).

Heat-shock and temperature-shift experiments

For heat shock, individual males were staged by examination of seam cells with Nomarski optics (Sulston and Horvitz, 1977). After staging, they were transferred to a siliconized Eppendorf tube containing 100 µl of M9 buffer (Brenner, 1974), which was placed in a circulating waterbath under the heat-shock conditions specified. After heat shock, animals were recovered, placed at 25°C on pre-equilibrated, seeded plates and allowed to develop to adulthood.

In the *daf-4ts* temperature-upshift experiments, animals grown at 15°C were staged as described above, then transferred to plates pre-equilibrated at 25°C. In the downshift experiments, synchronized populations derived from eggs laid during an interval were maintained at 20°C until the end of the L1 larval stage to avoid dauer formation. At the beginning of L2, plates were transferred to 25°C. Males developed at 25°C for at least 12 hours before being staged and transferred to plates pre-equilibrated at 15°C. In both heat-shock and *daf-4* temperature-shift experiments, adult males were scored for ray morphology by Nomarski optics and for GFP fluorescence. In selected experiments, animals were recovered from the slide and subjected to FIF assay.

RESULTS

Background

Development of the dopaminergic ray sensory neurons is summarized in Fig. 1. Ray cells arise after a series of postembryonic cell divisions from three pairs of embryonic neuroectoblast cells, V5, V6 and T, located in the posterior body and tail regions in the hatched L1 larva (Sulston and Horvitz, 1977). These postembryonic lineages generate nine pairs of ray precursor cells (Rn cells) in the L3 larval stage. Ray precursor cells divide following a stereotyped pattern, the ray sublineage, to generate the two sensory neurons and one glial-like structural cell of a single ray. The ray itself consists primarily of an extension of the syncytial hypodermis, hyp 7, which surrounds and contains the processes of the neurons and structural cell (Sulston et al., 1980; Chow et al., 1995). The two neurons are of two types defined by their ultrastructure (A-type and B-type) and are similar in all the rays (Sulston et al., 1980). The A-type neurons of rays 5, 7 and 9 contain DA (Sulston and Horvitz, 1977). One of the two neurons, the type

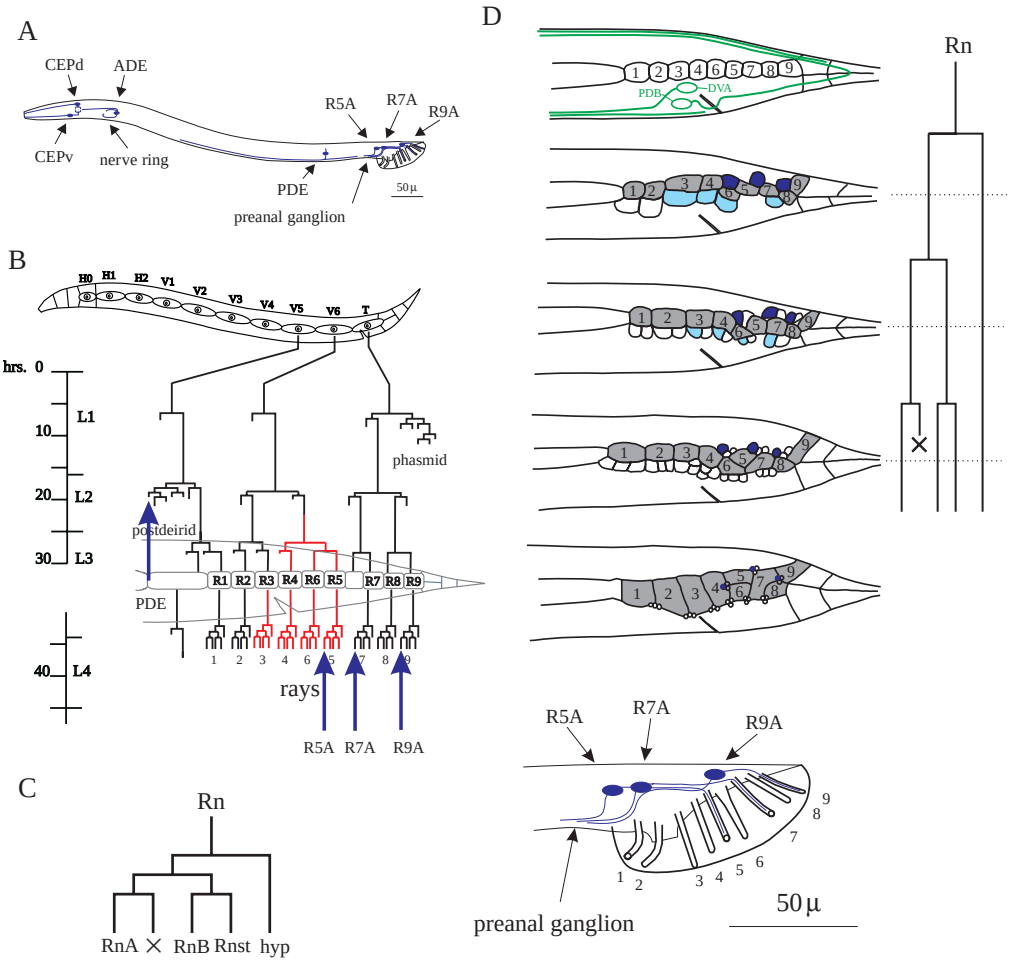

Fig. 1. Development of dopaminergic ray neurons. (A) Dopaminergic neurons in an adult male; lateral view showing one member of each bilateral pair. Dorsal CEP, ventral CEP, ADE and PDE neurons are also found in hermaphrodites (Sulston et al., 1975; White et al., 1986). (B) Postembryonic cell lineages leading to the ray neurons (Sulston and Horvitz, 1977). Hours of postembryonic development and larval stage are given on the scale to the left. The postdeirid and phasmid are sensory structures present in both sexes. R1-R9 are the ray precursor cells (Rn). Lineage branches expressing the *Hox* gene *egl-5* are shown in red. Blue arrows indicate dopaminergic cells. (C) Each Rn cell expresses the ray sublineage and generates the A-type neuron (RnA), B-type neuron (RnB), and structural cell (Rnst) of one ray, one cell death (×), and one hypodermal cell (hyp, the Rn.p). (D) Development of ray cells from the Rn cell stage onwards. The Rn cell and its progeny lie initially in the epidermal layer; the diagrams show the surface cell profiles of a component of adherens junctions (visualized by MH27 antibodies and by expression of the reporter gene, *jam-1::gfp*). Rn.p hypodermal cells are shown in gray and are numbered. Cells that generate a dopaminergic A-type neuron and the dopaminergic neurons themselves are dark blue; cells that can be induced by ectopic expression of the DBL-1 ligand to generate a dopaminergic neuron are in light blue. Two neurons, DVA and PDB, and the dorsal and ventral nerve cords, all of which have been shown to express a *dbl-1* reporter gene and hence could be the source of DBL-1 ligand, are shown in green in the top diagram. (*dbl-1* reporter genes are also expressed in the spicule cells, not illustrated (Morita et al., 1999; Suzuki et al., 1999).) After the sublineage is completed (fourth diagram), the cells begin to differentiate and their cell bodies move out of the epidermal layer, as shown by their sharply reduced adherens junction profiles (fifth diagram). The profiles of the Rn.p cells increase correspondingly. In a morphological reorganization at the end of the L4 larval stage, the posterior region retracts and the rays and acellular fan of the adult male extend (last diagram).

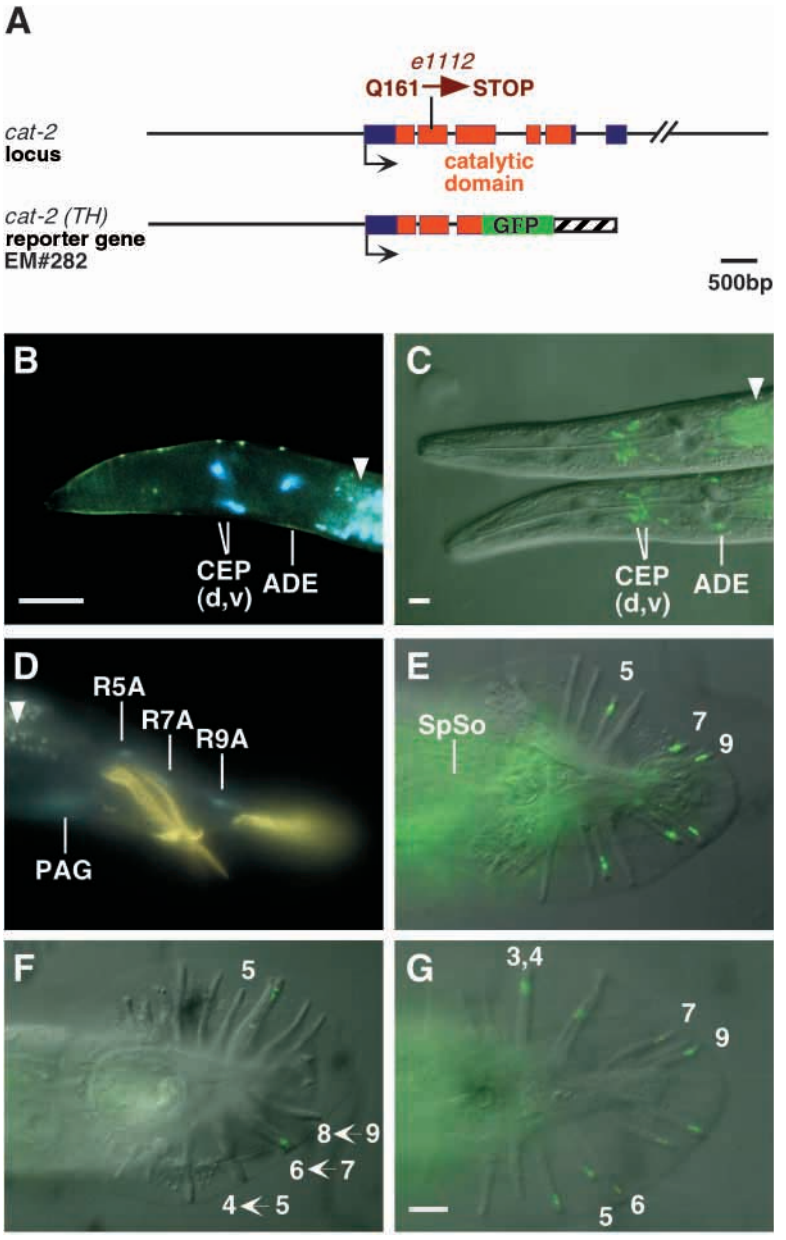

Fig. 2. Expression of the *cat-2::gfp* reporter in dopaminergic cells. (A) Structure of the *cat-2* (TH) locus and derived *gfp* reporter construct, EM#282. Hatched region is the 3′ UTR of *unc-54*, provided by the vector. (B) Rescue of *cat-2(e1112)* by microinjection of the cloned *cat-2* gene. Dopamine (light blue), visualized by FIF treatment, is in the cell bodies of a dorsal and ventral cephalic (CEPd/v) and a deirid (ADE) neuron of the head (cells identified by position) (lateral view, anterior to left in all photomicrographs). Arrowhead indicates autofluorescence associated with gut granules. (C) CAT-2::GFP expression in head neurons in wild-type animals transformed by EM#282 (dorsal view). (D) Adult male tail showing dopamine in ray neuron cell bodies and axon terminals (FIF, lateral view). Fluorescence of the axon terminals is seen in the preanal ganglion (PAG). Yellow fluorescence is autofluorescence associated with spicules and fan structures (FIF of the socket cells of the copulatory spicules is not visible in this focal plan). (E) CAT-2::GFP in the tail of a wild-type adult male (ventral view). Fluorescence in the rays just short of the tips is consistent with localization in the dendritic endings of the A-type neurons, which, unlike the dendritic endings of the B-type neurons, do not extend into the opening at the ray tip (Sulston et al., 1980; Chow et al., 1995). Fluorescence in the body is from the cell bodies of R5A, R7A and R9A and the socket cells of the copulatory spicules (SpSo). (F) CAT-2::GFP in a *dbl-1(wk70)* mutant male (ventral view). Several ray morphological abnormalities are apparent: on the left side, ray 7 is fused to ray 6; on the right side, ray 5 is fused to ray 4, ray 7 to ray 6 and ray 9 to ray 8. On both sides, CAT-2::GFP is absent from rays 5 and 9 but is retained in both 6-7 fused rays. (G) CAT-2::GFP expression in multiple rays after heat-shock-induced expression of a *HS::dbl-1* transgene (ventral view). A *dbl-1(wk70)* mutant animal carrying a mixed transgenic array generated by coinjection of the HS::*dbl-1* transgene and *cat-2::gfp* reporter EM#282 was induced by heat shock at the Rn stage. Heat shock rescued ray 5, 7 and 9 morphology and dopaminergic identity, and induced dopaminergic identity in additional rays (compare to F). Ectopically expressing rays often display altered ray morphology; here, on both sides ray 6 has adopted a ray 4-like morphology and on the left side rays 3 and 4 are fused (3,4). All scale bars = 10 μm; scale bar in G applies to D-G.

of which has not been determined, in rays 1, 3 and 9 contains serotonin (Loer and Kenyon, 1993).

The *cat-2* locus encodes *C. elegans* TH

In order to study specification of dopaminergic cell fate in rays 5, 7 and 9, we identified the gene for tyrosine hydroxylase (TH), which catalyzes the initial and rate-limiting step in DA biosynthesis, and constructed *gfp* reporters. We showed that the predicted gene B0432.5 corresponds to the previously identified DA-deficient locus *cat-2*. B0432.5, which encodes a predicted polypeptide with 50% amino acid identity to *Drosophila* and rat tyrosine hydroxylases (Grima et al., 1985; Neckameyer and Quinn, 1989), and which maps in the vicinity of *cat-2*, rescues the DA-deficient mutant phenotype of *cat-2(e1112)* when injected into *e1112* animals (Fig. 2B). Furthermore, in a strain homozygous for *cat-2(e1112)*, B0432.5 contains a stop codon within the predicted TH catalytic domain that would be expected to eliminate gene function, demonstrating that *cat-2* encodes TH (Goodwill et al., 1997) (Fig. 2A).

We constructed *cat-2::gfp* reporter genes by inserting a *gfp* cassette at several points within the *cat-2* open reading frame. All reporters generated the same expression pattern. The reporter used most extensively and illustrated here (EM#282) consists of *gfp* appended to the third exon of a truncated *cat-2* gene driven by 1.5 kb of upstream genomic sequence (Fig. 2A). In wild-type worms, expression of *cat-2::gfp* reporters was restricted to the 14 cells previously identified by a formaldehyde-induced fluorescence (FIF) assay for DA (Sulston et al., 1975), plus 4 additional male-specific FIF-positive cells identified here for the first time (Figs 1A, 2). These are the four socket cells of the spicules (kindly identified by L. Jiang and P. W. Sternberg). We confirmed in selected mutant backgrounds that, as in wild type, reporter gene expression was consistent with the pattern of FIF. Thus, the

cat-2::gfp reporter specifically marks dopaminergic cells and can be used as a convenient indicator of DA expression.

Adoption of the dopaminergic fate is accompanied by activation of the TH gene in an all-or-none fashion

In all of the genetic backgrounds examined, cells that expressed the *cat-2::gfp* reporter did so at a level similar to the level in cells of wild-type animals. The developmental mutations studied appeared to alter the probability of adoption of the dopaminergic cell fate, and not the nature of the dopaminergic cell state itself. Mutant backgrounds in which expression of the dopaminergic cell fate was altered were characterized by complete loss of fluorescence in some cells, whereas corresponding cells on the contralateral side or in other animals had normal levels of fluorescence, both of CAT-2::GFP and FIF (based on visual estimation). We interpret this all-or-none response to indicate that the mutations described below affect the adoption by a cell of a particular transcriptional cell state. We used CAT-2::GFP expression and FIF assays to study the genetic requirements for adoption of this transcriptional state, and report the data in terms of the percentage of cells expressing.

CAT-2::GFP fluorescence first appeared in ray neurons R5A, R7A and R9A shortly after they were born (data not shown). Therefore, transcriptional activation of *cat-2* most likely occurs in postmitotic neurons after the ray sublineage is complete at the time when the ray neurons begin to differentiate. GFP fluorescence persisted in mature ray neurons of the adult male (Fig. 2E). Fluorescence was present in the cell body and in both axonal and dendritic processes, but tended to be concentrated, for unknown reasons, in the ray tips. This fortuitous property allowed us to assign the dopaminergic fate to specific rays. This is not possible by FIF assay, since FIF labels the variably situated ray cell bodies and the axon processes, but not the dendrites of the ray neurons.

The DBL-1 signaling pathway patterns dopaminergic fate among ray neurons

The DBL-1 signaling pathway is required for specification of the morphogenetic identities of a subset of the rays that includes the dopaminergic rays, rays 5, 7 and 9 (Baird et al., 1991; Savage et al., 1996; Krishna et al., 1999; Morita et al., 1999; Suzuki et al., 1999). We investigated whether the same signal helps to define ray neurotransmitter identity. We studied the effects on expression of *cat-2::gfp* reporter genes and, in selected instances, on FIF, of single, double and triple loss-of-function mutants involving all known components of this pathway (Fig. 3 and data not shown).

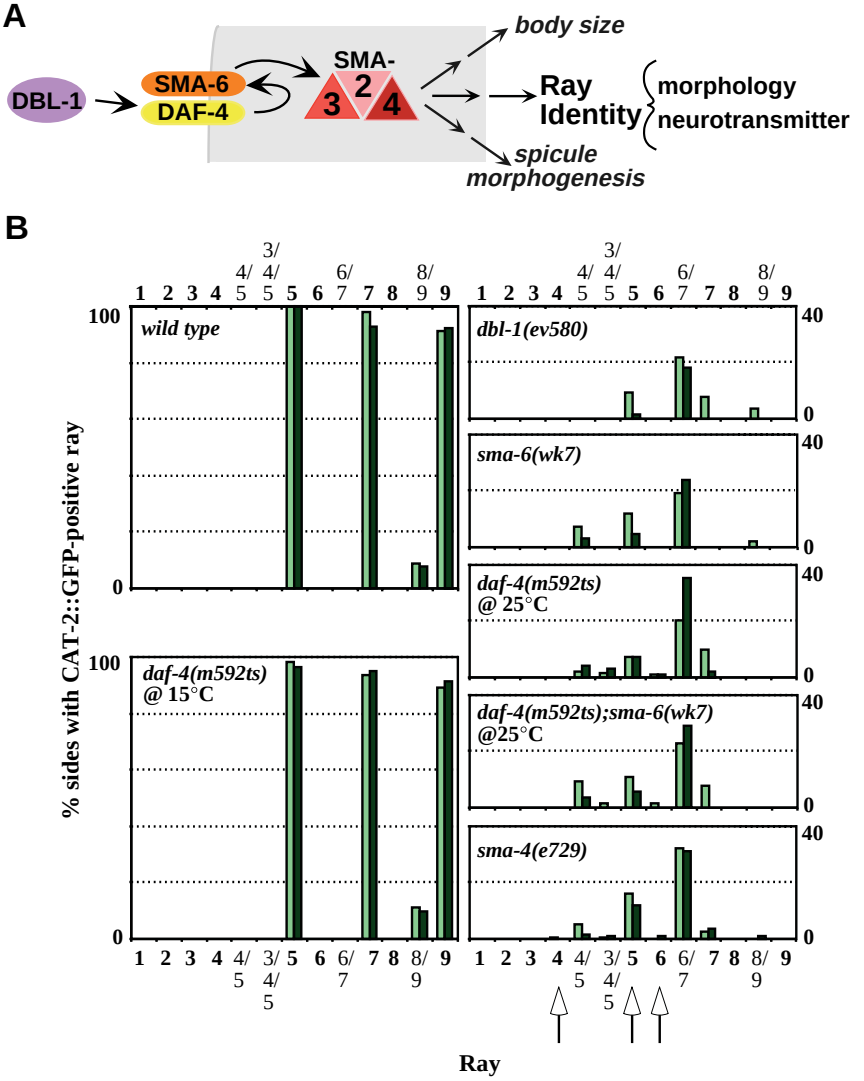

Fig. 3. Expression of the dopaminergic fate in ray neurons in DBL-1 pathway mutants. (A) The DBL-1 pathway in the male tail (see Introduction). (B) Percentage of rays expressing CAT-2::GFP in wild type and DBL-1 pathway mutants. Selected examples are shown. The following single, double and triple mutant combinations were examined: *dbl-1(ev580), dbl-1(wk70), sma-6(wk7), sma-6(wk8), daf-4(m592ts), sma-6(wk7);daf-4(m592ts), sma-2(e502), sma-2(e172), sma-3(e491), sma-3(e637), sma-4(e729), sma-2(e502)sma-3(e491)* and *sma-2(e502)sma-3(e491)sma-4(e729)*. Of these, *dbl-1(ev580), dbl-1(wk70), sma-6(wk7), sma-6(wk8) and sma-3(e637)* are presumptive null or severe loss-of-function mutations (Suzuki et al., 1999; Krishna et al., 1999). Results for two independently generated extrachromosmal arrays are shown (*bxEx35:* green, *bxEx36:* olive). Rays are identified by their morphology, position and the position on the fan where their tip opens (scored as dorsal, ventral, or marginal). Lineages on each side of a single animal appear to develop separately and so sides are scored independently. In DBL-1 pathway mutants, rays are often fused (a low level of 8-9 fusion seen here in wild-type background and in *daf-4(m592ts)* at 15°C occurs also in untransformed wild-type strains). Fused rays contained only a single CAT-2::GFP-positive neuron. Because it was not possible to assign the positive cell to one ray within the fusion, fused rays were scored separately. The number of sides scored for each array in a given mutant background ranged from 60 to 144. The pattern of FIF was examined in each mutant (20-40 sides) and found to be consistent with the results shown for the reporter gene (data not shown). Open arrows indicate rays showing a discordance between ray morphology and dopaminergic identity (see Discussion).

In the absence of DBL-1 pathway signaling, the dopaminergic fate was still adopted by ray neurons, but far less consistently than in wild type (Figs 2F, 3B). In contrast, expression of DA by the non-sex-specific dopaminergic neurons was unaffected (data not shown). The results were similar for all mutations, including several that are likely to be null mutations, as well as combinations of mutations. They can be summarized as follows. For the normally dopaminergic rays 5, 7 and 9, the frequency of CAT-2::GFP expression and FIF was reduced from over 95% in wild type to 10%-20% for ray 5, 20%-40% for ray 7, and 0-2% for ray 9. (Levels lower than 100% in wild type can be accounted for by mosaic loss in the case of the unintegrated reporter transgene, and by incomplete permeabilization of worms in the FIF assay.) In addition, CAT-2::GFP fluorescence was present at low frequency in rays 4 and 6, rays that were never observed to adopt dopaminergic fate in wild type (combining the data from all mutants, 4/1227 rays with unambiguous ray 4 morphology were positive and 10/369 rays with unambiguous ray 6 morphology were positive; in wild type, 0/1041 rays with ray 4 morphology and 0/1041 with ray 6 morphology were positive; Fisher's exact test P-value for ray 4 is 0.13, for ray 6 is 10^{-6}). Rays 1, 2, 3 and 8 were not observed to express the *cat-2::gfp* reporter or to have FIF in any loss-of-function background (*n*=1818 sides scored totaling all mutants). Fused rays, scored separately, also sometimes contained a single CAT-2::GFP-positive neuron. The results indicate that DBL-1 signaling increases the probability that the dopaminergic fate will be adopted by rays 5, 7 and 9, whereas it appears to decrease adoption of dopaminergic fate by ray 6 and possibly also by ray 4.

Expression of the dopaminergic fate in DBL-1 pathway mutants is unlikely to be due to residual activity of the DBL-1 pathway for several reasons. First, whereas residual pathway activity might be expected to vary from one mutant to another, all DBL-1 pathway mutants gave DA expression profiles that were essentially the same. Second, disrupting two or three pathway components simultaneously gave the same pattern as single mutants. Third, some of the mutations that we used are predicted molecular nulls (Fig. 3 legend). Since the genome appears to encode only a single recognizable gene for a Type II TGFβ receptor (*daf-4*), a mutation in this gene would appear to preclude activation of the SMAD signal-transduction pathway by any of the other ligand, type I receptor, or SMAD genes encoded by the genome. Mutations in the additional TGFβ-pathway genes *daf-1, daf-3, daf-8* and *unc-129*, which are known from their effects on other tissues, were examined and had no effect on FIF in the rays (data not shown). We conclude that DA expression by ray neurons in DBL-1 pathway mutants either reflects an intrinsic property of these cells, or is induced by a signal unrelated to TGFβ. A third possibility is that it is induced by a TGFβ-family signal that acts via an as yet unrecognized receptor.

The reduced frequency of dopaminergic ray cells in DBL-1 pathway mutants is not due to a general failure of A-type neurons to differentiate. Rays 5, 7 and 9 have normal ray lineages, indicating that all cells of these rays are generated (Savage et al., 1996; Suzuki et al., 1999; this laboratory, unpublished observations). Furthermore, all rays are present, albeit with abnormal morphology and often fused together. Ray development implies the ray structural cell, which is necessary for ray extension, is generated and has differentiated (Sulston and Horvitz, 1977). To confirm that ray neurons differentiate, we examined expression of an *osm-6::gfp* reporter gene in *sma-4(e729)* and *sma-6(wk7)*. *osm-6* encodes a component of the ciliary apparatus of sensory neurons, and is expressed in both A-type and B-type ray neurons (Collet et al., 1998; D. S. Portman and S.W. E., unpublished data). *osm-6::gfp* expression was unaffected by the *sma-4* and *sma-6* mutations (data not shown), indicating the DBL-1 pathway is not necessary for all aspects of ray neuron differentiation.

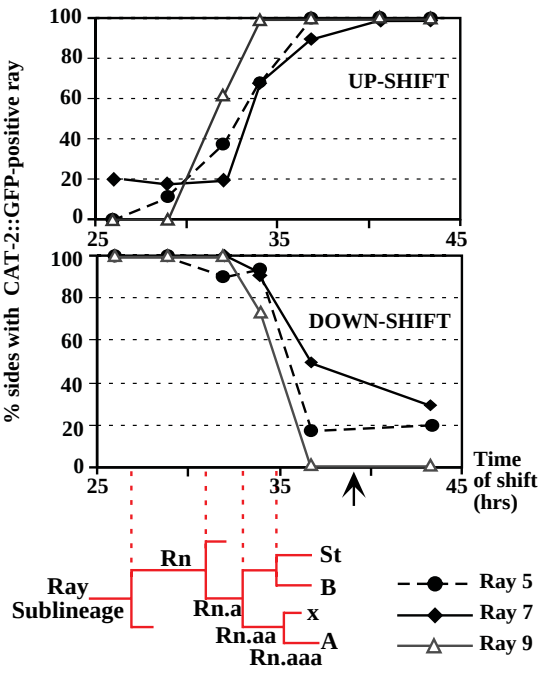

Fig. 4. Time of action of the DBL-1 pathway. At the stage of the ray sublineage shown, L3 or L4 *daf-4(592ts)* males carrying a *cat-2::gfp* extrachromosomal transgenic array (*bxEx35* or *bxEx36*) were shifted either from 15°C to 25°C (upshift) or from 25°C to 15°C (downshift) and allowed to mature to adults. Animals were scored as adults for ray expression of CAT-2::GFP and ray morphology, and in selected cases were stained for dopamine by FIF. The number of sides scored at each stage for upshift and downshift, respectively, was as follows: before Rn, 12, 10; Rn, 16, 18; Rn.a, 22, 16; Rn.aa, 24, 8; 38 hours (ray cell groups formed but undifferentiated), 6, 10; 41 hours (upshift only, ray cell differentiating, tail undergoing morphogenesis) 10, 0; 44 hours (adult), 10, 16. Arrow indicates earliest time at which CAT-2::GFP can be detected in ray neurons.

The DBL-1 pathway acts during the ray sublineage

To determine when the DBL-1 signal acts to influence expression of the dopaminergic fate, we carried out temperature-shift experiments with a temperature-sensitive allele of the type II receptor gene, *daf-4(m592ts)* (Fig. 4). The upshift experiment indicates that most induction normally occurs in the Rn.a cell. Animals raised to the nonpermissive temperature of 25°C after the Rn.a cells have divided are nearly fully induced. The downshift experiment shows, however, that induction can occur later, after the Rn.a cells have divided, in the Rn.aa cells. Thus, animals lowered to the permissive temperature before the Rn.aa cells divide are also nearly fully induced. However, once the Rn.aa cells have divided, the efficiency of induction falls rapidly and the differentiating neurons themselves are essentially

refractory. Thus there is an optimal window in the Rn.a and Rn.aa cells when the DBL-1 pathway is effective. The upshift experiment also shows that, once induction occurs, the signal is no longer required for the ray neurons to express the *cat-2::gfp* reporter. When worms are moved to and maintained at high temperature after induction has occurred, the *cat-2::gfp* reporter comes on when the neurons begin to differentiate, up to 10 hours or two cell divisions after temperature shift. It seems likely that the action of the DAF-4 receptor and SMAD signal transduction pathway is in the ray neurons or their precursors, as it is for ray morphogenesis (Savage et al., 1996). However, it remains possible that DAF-4 acts in another cell at the times defined in this experiment and that DA induction occurs via a secondary signal.

Ectopic expression of the DBL-1 ligand causes additional rays to adopt dopaminergic fate

In the temperature-shift experiments above, failure of postmitotic cells to be induced after late downshift might be because there is no ligand present at this late time or, alternatively, because differentiating neurons are no longer competent to respond or are prevented from responding by a DBL-1 antagonist. The limited information regarding the expression of DBL-1 in wild type, from reporter gene studies, is insufficient to resolve this issue. Reporter genes are expressed in two identified tail neurons and in the dorsal and ventral nerve cords throughout postembryonic development and adulthood (see Fig. 1D) (Morita et al., 1999; Suzuki et al., 1999). The spatial distribution of the DBL-1 protein is unknown, and functional studies have not yet identified which, if any, of these putative sources contributes to patterning the rays (Y. Suzuki and W.B Wood, personal communication).

To distinguish between the alternatives, lack of ligand versus inability to respond, as well as to study the role played by ligand level and distribution in patterning the dopaminergic fate, we supplied DBL-1 from a heat-shock transgene. The heat-shock promoter used results in inducible, ubiquitous expression at a high level in many or all tissues (Russnak and Candido, 1985; Stringham et al., 1992; Salser and Kenyon, 1996). Heat-shock induction experiments were carried out in a *dbl-1* mutant background to eliminate the endogenous source of ligand. Therefore, the onset of ligand expression was controlled by the timing of heat shock.

Rays could be induced to express CAT-2::GFP by heat shock from the earliest time tested, corresponding to the cell division prior to Rn, until a few hours after the end of the ray sublineage (Fig. 5). Once ray neurons were born, their ability to be induced declined, and after 6 hours most became refractory. Later heat shock of young adults had no affect. Thus, competence declines rapidly or there is an inhibitor once ray neurons begin to differentiate.

This experiment also allowed us to address the role played by regulation of DBL-1 expression in establishment of the spatial pattern of dopaminergic neurons among the rays. If regulation of DBL-1 expression was important for establishment of DA expression only in rays 5, 7 and 9 (for example, if expression is restricted to a specific zone or a critical level), then expression of *dbl-1* from the heat-shock promoter might result in a different DA expression pattern from wild type. However, if the ligand merely served to trigger an already established prepattern such that only rays 5, 7 and 9 were capable of responding, then alteration of ligand expression would not affect the DA expression pattern.

We found that after heat shock in some animals there were more than three CAT-2::GFP-positive cells per side (Fig. 5). Significant CAT-2::GFP expression occurred in rays 3, 4, 6 and 8, and in fusions involving these rays, in addition to rays 5, 7 and 9 (Figs 2G, 6). Similarly, animals carrying multiple *dbl-1(+)* transgenes resulted in more than three FIF-positive rays per side (Suzuki et al., 1999; R. L. and S. W. E., unpublished observations). These results establish that rays 3-9 are all competent to respond to DBL-1 induction and suggest that regulation of ligand expression is important in confining the response to rays 5, 7 and 9.

Expression of dopaminergic fate in additional rays after heat shock might have been because an abnormally high level of ligand overcame the prepattern, because the heat-shock transgene generates DBL-1 in a novel spatial pattern (presumably ubiquitously), or for both of these reasons. In order to determine whether a low level of presumably ubiquitous ligand could trigger a normal pattern of DA expression, we varied the extent of heat shock during the Rn cell stage (Fig. 6). Under the mildest heat-shock conditions, CAT-2::GFP was expressed at nearly 100% frequency in rays 5, 7 and 9, but was still observed at low frequency in rays 3, 4, 6 and 8. Therefore, much of the pattern is due to prepatterned differences in response of the rays. Yet it appears that the heat-shock transgene cannot completely reproduce the wild-type pattern. This suggests that spatial restriction of the expression pattern of DBL-1 may also contribute to patterning the rays. However, since we do not know the level of DBL-1 generated in any of these experiments, we cannot rule out the possibility that all these effects are due to higher than wild-type ligand levels.

Increase of the duration and temperature of heat shock increased the frequency of CAT-2::GFP expression to as much as 65% in ray 4 and 40% in ray 3. In contrast, rays 1 and 2 could not be induced under any heat-shock conditions. Thus

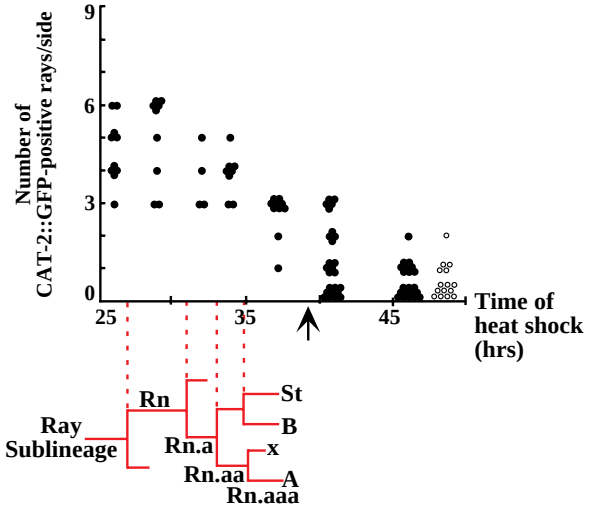

Fig. 5. Induction of the dopaminergic fate by expression of ligand from a heat-shock promoter at various stages. Animals were heat shocked at 33°C for 30 minutes at the stages shown. Each circle represents one side scored: filled circles are animals subjected to heat shock, open circle are animals not subjected to heat shock. Arrow indicates earliest time at which CAT-2::GFP can be detected in ray neurons.

there are at least three types of rays, those that are induced with high frequency (rays 5, 7 and 9), those that can be induced with moderate frequency (rays 3, 4, 6 and 8), and those that cannot be induced. One explanation is that prepatterning produces a complex pattern of ray characteristics. The wild-type pattern of DA expression appears to derive from the cumulative effects of all three factors: the amount of ligand, the spatial distribution of ligand and a variable sensitivity of prepatterned rays to respond. However, there may also be effects due to additional positively or negatively acting signals.

The *Hox* gene *egl-5* is necessary for rays 3-5 to adopt the dopaminergic fate

We noted that, among V rays (rays 1-6), competence to express DA was correlated with expression of the *Hox* gene *egl-5* (Ferreira et al., 1999) (Fig. 1B,D). EGL-5 is present in the lineages leading to rays 4-6, and is also expressed in R3 and its progeny. All of these rays can be induced to express DA, whereas rays 1 and 2 do not express EGL-5 and cannot be induced. We therefore tested whether *egl-5* was necessary for establishment of the competent state among V rays. We found that, in an *egl-5* loss-of-function mutant, CAT-2::GFP fluorescence and FIF were not observed in ray 5 but were unaffected in rays 7 and 9 (Fig. 7 and data not shown). Expression of the *osm-6::gfp* reporter showed that all the ray neurons except those of ray 6 were present as expected. Therefore *egl-5* is necessary for adoption of dopaminergic fate by the R5A neuron in wild type.

In double mutants defective in both *egl-5* and DBL-1 pathway function, CAT-2::GFP was not expressed in ray 5 or in ray 4, but was expressed in rays 7 and 9 at the same frequency as in DBL-1 pathway single mutants (Fig. 7B). After heat-shock induction of DBL-1 in an *egl-5* mutant background, there was essentially no expression of CAT-2::GFP by rays 3, 4 and 5 (Fig. 7B). Therefore *egl-5* is necessary for both DBL-1 pathway-dependent and DBL-1 pathway-independent expression of DA by rays descended from the blast cell V6. It is not necessary for expression of DA by rays descended from the blast cell T.

DISCUSSION

The mechanism of patterning dopaminergic fate among ray neurons

We have studied the patterning mechanism that results in expression of the neurotransmitter dopamine by three of nine RnA sensory neurons. Our main conclusions are that a *Hox* gene and a TGFβ-family signaling pathway cause ray neurons adopt the dopaminergic fate in an apparently all-or-none fashion: the *Hox* gene gives certain ray neurons the capacity to adopt this fate and the TGFβ-family signal influences the probability that a neuron does so.

The patterning mechanism appears to consist of two steps. The temporal order of these two steps is unknown. In a prepatterning step, 7 out of 9 progenitor cells (leading to rays 3-9) are made competent to generate dopaminergic neurons. The prepattern mechanism excludes two of the rays (rays 1 and 2), which were essentially never observed to express dopamine. In the second step, the DBL-1 signaling pathway alters the probability that dopaminergic fate will be adopted by competent cells. We found that the *Hox* gene *egl-5* is necessary to establish the competence of rays 3-6. EGL-5 protein is present in the lineages leading to rays 4-6 before the Rn cell stage, when the DBL-1 signal acts, and first appears in the Rn cell in the ray 3 lineage (Ferreira et al., 1999). Therefore it is natural to suppose that the prepattern is established before or at the same time as the inducer acts. However, we have no data bearing on the time of action of EGL-5 and its action might be required after that of the DBL-1 pathway.

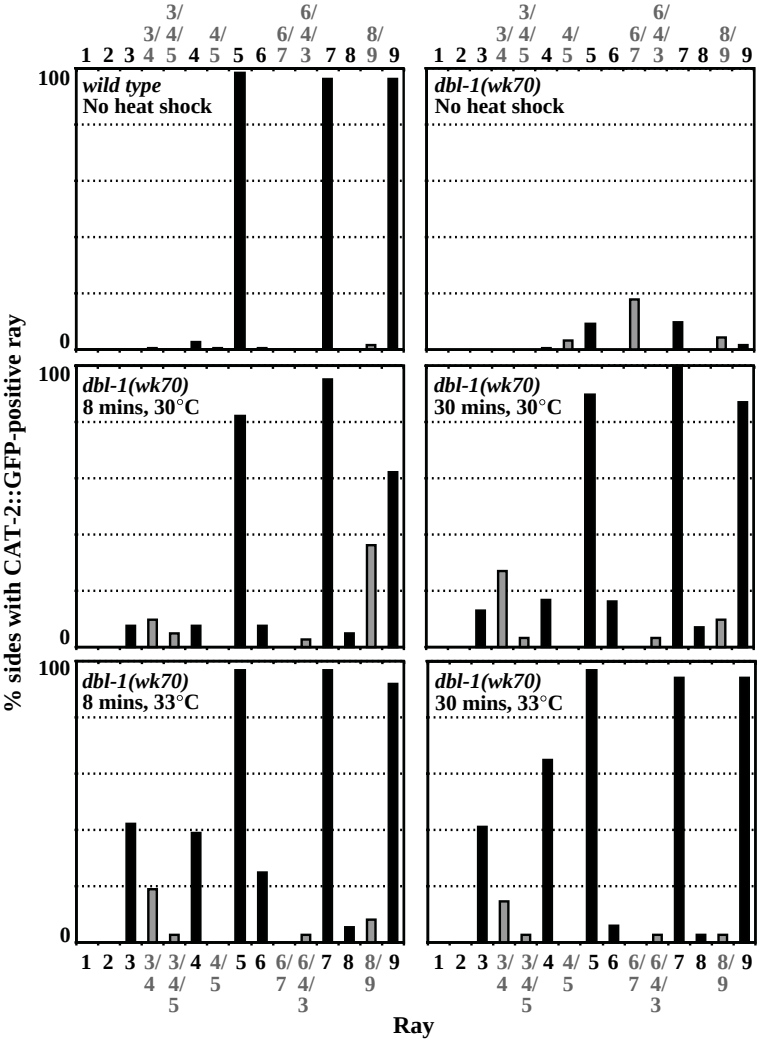

Fig. 6. Effect of varying heat-shock conditions on induction of the dopaminergic fate. Wild-type or *dbl-1(wk70)* males containing mixed HS::*dbl-1*/*cat-2::gfp* extrachromosomal transgenic arrays (*bxEx46* or *bxEx47*) were subjected at the Rn cell stage to the heat-shock regimens shown. The number of sides scored for each condition was as follows: no heat shock, 108 for *dbl-1(+)* (wild type), 221 for *dbl-1(wk70)*; 8 minutes at 30°C, 39; 30 minutes at 30°C, 30; 8 minutes at 33°C, 36; 30 minutes at 33°C, 34. Data for fused rays are shown in gray for clarity. Expression of the reporter in rays other than rays 5, 7 and 9 in the absence of heat shock is dependent on presence of the HS::*dbl-1* transgene (data not shown) and is presumably due to leakiness of the heat-shock promoter.

When ligand is supplied from a heat-shock transgene, all competent cells can be induced at varying frequencies to generate dopaminergic neurons. In the absence of ligand or if the signal transduction pathway is blocked by mutation, at low to moderate frequencies some cells within the competent group generate dopaminergic neurons apparently spontaneously. The DBL-1 signal is therefore not necessary for expression of the dopaminergic phenotype, but functions in generation of a robust pattern.

Our genetic results do not prove that in regulating the dopaminergic phenotype *egl-5* and the DBL-1 pathway act cell-autonomously within the ray neurons and their precursors. However, both are known to be present within these cells. EGL-5 has been detected by antibody staining (Ferreira et al., 1999) and mosaic analysis has demonstrated cell-autonomy of *egl-5* action in ray generation and morphogenesis (Chisholm, 1991). Likewise, mosaic analysis has demonstrated cell-autonomy of the DBL-1 receptor *daf-4* and the SMAD signal transduction pathway in ray morphogenesis (Savage et al., 1996).

In the context of this study, activation in ray neurons of reporters for the gene encoding tyrosine hydroxylase, *cat-2*, as well as expression of DA determined by histochemical assay, appeared to occur in an all-or-none fashion. This suggests that expression of the *cat-2::gfp* reporter reflects the adoption by a cell of a particular transcriptional cell state. On-or-off transcriptional behavior has been observed in other systems and can be interpreted in terms of the assembly of a stable transcription complex on target genes (e.g. Weintraub, 1988). Our data might reflect the genetic requirements for the assembly of such a complex on the *cat-2* promoter.

Because the expression of DA might represent one outcome of a choice between two or more states, it will be of interest to determine whether a normally dopaminergic cell that fails in regulatory mutants to acquire dopaminergic identity has instead adopted an alternative state, possibly involving expression of a different neurotransmitter, such as serotonin (Loer and Kenyon, 1993). Similarly, does a cell that inappropriately adopts dopaminergic identity switch off its normal neurotransmitter? Alternative, mutually exclusive cell states might explain why some cells, though competent to adopt the dopaminergic state, could not be induced to express DA at a frequency of 100%, even

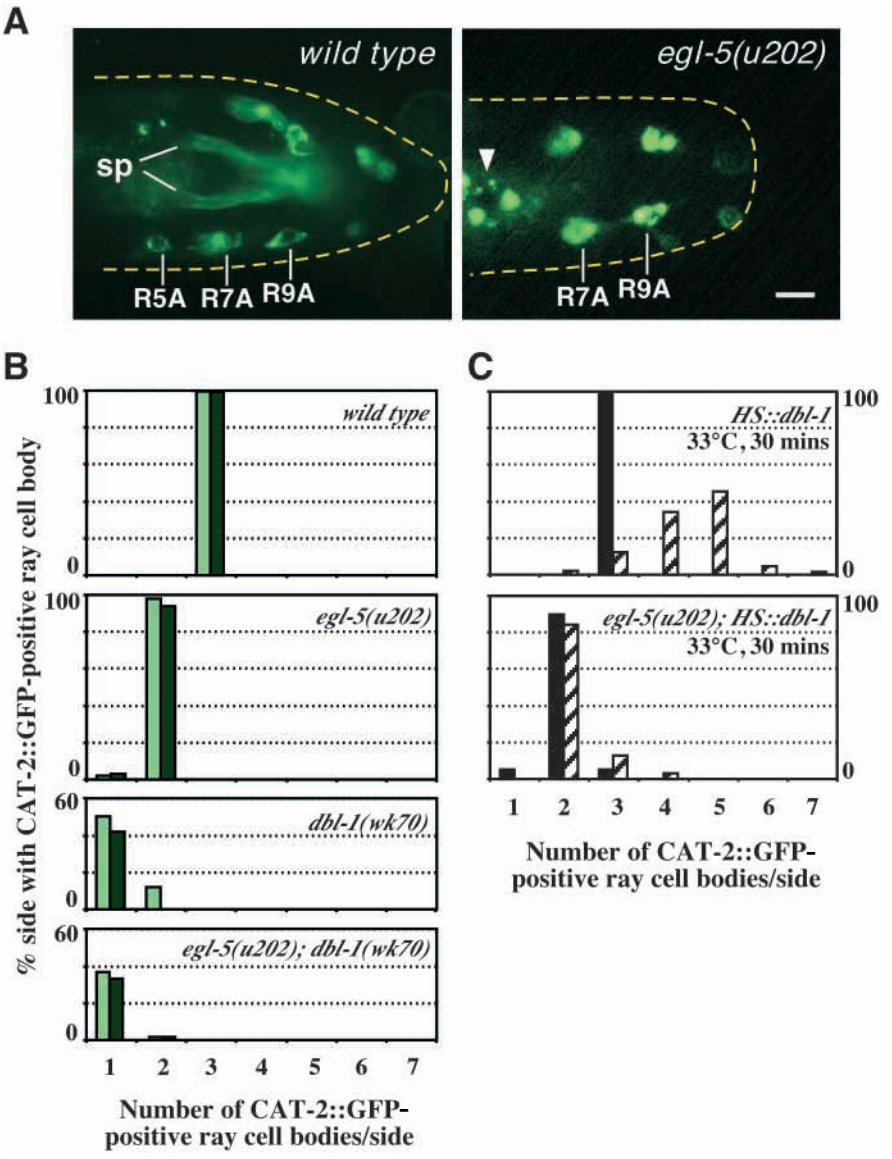

Fig. 7. Expression of dopaminergic fate in *egl-5* mutants. (A) CAT-2::GFP (EM#282) expression in ray neuron cell bodies of wild type (rays 5, 7 and 9) and *egl-5(u202)* (rays 7 and 9) (body outline is indicated by the dashed yellow line, dorsal view, anterior left, scale bar = 10 μm). In wild type, spicule autofluorescence (sp) is apparent. In *egl-5*, spicules are absent, the arrowhead indicates gut autofluorescence and fluorescent areas posterior of the R9A cell body are the tips of ray 9. (B) Expression of CAT-2::GFP in wild type, *egl-5*, *dbl-1* and *egl-5 dbl-1* double mutants. Results are shown for two independently generated extrachromosmal arrays (*bxEx35*, green; *bxEx36*, olive). Because *egl-5* blocks normal ray development, the number of ray cell bodies rather than rays is scored. n values for each extrachromosomal array in each mutant background ranged from 71-128. (C) Expression of CAT-2::GFP in wild type and in *egl-5* mutants after heat-shock induction of the *dbl-1* transgene (hatched bars: heat-shocked animals, black bars: no heat shock). Wild-type animals and *egl-5(u202)* mutant males carrying the HS::*dbl-1/cat-2::gfp* extrachromosomal arrays (*bxEx46* or *bxEx47*) were subjected to 30 minutes heat shock at 33°C at the Rn stage. In *egl-5* mutants after heat shock, three cell bodies per side corresponded to cells likely to be neurons of rays 7, 8 and 9 (judging by cell position), whereas 4 cell bodies per side were likely to be neurons of rays 5, 7, 8 and 9 (however, identification of the most anterior fluorescent cell as the cell body of R5A is made uncertain by the occasional (5%) appearance in an *egl-5(u202)* background of additional, unidentified fluorescent non-ray neurons – data not shown). Data from the two independently generated transgenic lines did not differ significantly and are combined. n values for heat-shock-treated animals (hatched): wild type, 61; *egl-5(u202)*, 57; no heat-shock treatment (black): wild type, 100; *egl-5(u202)*, 50.

at the highest levels of induction in heat-shock experiments. A possible explanation is that some of these cells have already adopted an alternative, refractory state and cannot be induced. Thus these studies might define factors affecting one or more switches between states or pathways.

Nature of the prepattern

All competent ray cells were not alike with respect to either spontaneous expression or induction of DA. Therefore there is no single competent state. In the absence of a DBL-1 pathway signal, the probability among competent cells of spontaneous switching to DA expression varied from as low as 1% (ray 4 or 6) to as high as 35% (ray 7). Likewise, the sensitivity to induction by ectopic ligand varied, being highest in the precursors of the three normally dopaminergic neurons. Among competent precursors of normally non-dopaminergic ray neurons, the response to high levels of heat-shock induction varied from 10% (ray 8) to 65% (ray 4). Apparently, patterning events during the cell lineage generate a complex pattern of cells with variable intrinsic properties. This conclusion must be accepted with the reservation that some differences observed between competent cells could be due to uneven distribution of ligand in the heat-shock experiments, or to unequal exposure to additional signals, either DBL-1 antagonists or unknown signals that induce DA expression.

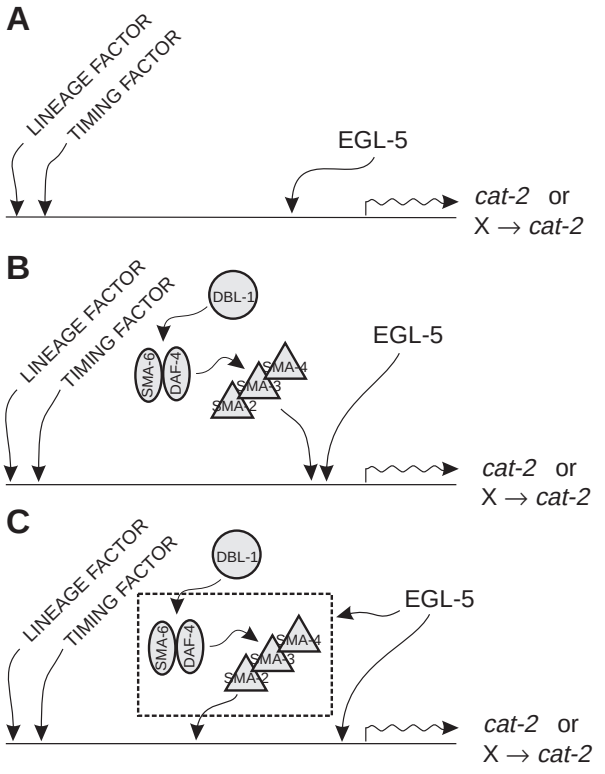

Fig. 8. Models to explain the respective functions of the DBL-1 pathway and *egl-5*. (A) Regulation of *cat-2* by EGL-5 in the absence of a DBL-1 signal. EGL-5 acts on the promoter of the *cat-2* gene itself, or on some gene, denoted by X, that is upstream of *cat-2*. Factors restricted respectively to the Rn.aaa sublineage branch (lineage factor) and to the postmitotic period of cell differentiation (timing factor) regulate EGL-5 activity at the same or a downstream step. (B,C) Two models to explain the relationship between EGL-5 and the DBL-1 signal. In B, by binding at a site nearby, activated SMAD proteins act only by enhancing the action of EGL-5 at its target promoter. In C, the activated SMAD proteins act independently of EGL-5 at a separate site. To explain the requirement for EGL-5 in DBL-1 pathway induction under this model, it is suggested that EGL-5 is necessary for synthesis of one or more components of the signal transduction pathway (dashed box).

Some of the patterning events that generate the prepattern involve regulation of expression of the *Hox* gene *egl-5*. EGL-5 is present in the lineage branches leading to rays 3-6 (Fig. 1B), and is necessary for the competence of rays 3-5 (and possibly also ray 6, but this could not be determined in the present experiments because *egl-5* is also necessary for generation of this ray). Expression of *egl-5* in these cells requires the *Hox* gene *mab-5*, itself subject to complex regulation in seam lineages (Salser and Kenyon, 1996; Ferreira et al., 1999). Thus, regulated expression of *Hox* genes contributes to generation of the prepattern and hence to the DA expression pattern. Genes playing a corresponding role in the rays 7-9, descended from blast cell T, are unknown.

Variability among prepatterned cells might result from variable levels of EGL-5 (Ferreira et al., 1999), or from differential actions of other cell-intrinsic factors or extracellular signals. For example, a form of the *C. elegans Pax-6* homolog, MAB-18, is expressed in ray 6 and is necessary for the morphological identity of this ray (Zhang and Emmons, 1995; Zhang et al., 1998). Thus, additional genes may contribute to the prepattern and help to explain its complexity.

Function of *egl-5* in the ray developmental pathway

One question of interest is whether pathways leading to the adoption of the neuronal cell fate are entirely independent of those that pattern individual neuronal properties. Several lines of evidence indicate that *egl-5* acts in specification of the ray neuroblast fate not only in ray 6, but also redundantly with *mab-5* in other V-rays (Chisholm, 1991; Salser and Kenyon, 1996; Ferreira et al., 1999). *egl-5* also acts in specification of ray morphology (Chisholm, 1991; Chow and Emmons, 1995) and, as shown here, in specification of DA expression. Since a single regulatory factor acts both to specify the neuronal cell fate and also the differentiated properties of individual neurons, this demonstrates that there are steps in common between the developmental pathways leading to expression of pan-neural genes and pathways leading to expression of neuron-specific genes. Similar multiple roles and times of action have been demonstrated for *mab-5* in posterior hypodermal cell lineages (Salser and Kenyon, 1996) and for the *Hox* gene *lin-39* in vulva development (Clark et al., 1993; Clandinin et al., 1997; Maloof and Kenyon, 1998).

In its late action, EGL-5 could act directly on the *cat-2* promoter or alternatively on the promoter of another transcription factor at some level in the hierarchy upstream of *cat-2* (Fig. 8). Additional intermediate transcription factors might include those of the LIM and POU families, which play widespread roles in *C. elegans* and in other organisms in specifying the properties of individual neurons (e.g. Way and Chalfie, 1988; Finney and Ruvkun, 1990; Thor and Thomas, 1997; Hobert et al., 1999). A transcription factor of the POU family regulates dopamine decarboxylase gene expression in *Drosophila* (Johnson and Hirsh, 1990).

Since EGL-5 is expressed in all branches of the ray

sublineages of rays 3 to 6, additional factors must intervene to direct its action in the dopaminergic pathway exclusively to the lineage branch leading to the A-type neuron. Likewise, turn-on of *cat-2* appears to be delayed until the ray sublineage is completed. Whether lineage-branch-specific and timing cues are integrated by the promoter of the *cat-2* gene itself, or by the promoters of intermediate transcription factors acting between *egl-5* and *cat-2,* is an interesting question for future studies.

Function of the DBL-1 pathway signal

The DBL-1 signal acts to alter the probability that an EGL-5-containing lineage branch will generate a neuron that expresses DA. How does this alteration of EGL-5 action occur? One possibility is that activated SMAD proteins act on target sites overlapping with or adjacent to EGL-5 binding sites. Upon binding, they might increase the probability that a stable transcription complex involving EGL-5 is formed or promote EGL-5 action in a transcription complex (Fig. 8B). Alternatively, activated SMAD proteins could act at a separate promoter site, possibly together with other, unknown factors, to provide an independent, parallel pathway to the dopaminergic state (Fig. 8C). Since *egl-5* is necessary for DBL-1 pathway-mediated induction, this second model requires that *egl-5* must make cells competent to receive the DBL-1 signal, for example, by transcribing one or more genes of the signal transduction pathway. These alternative models are not exclusive and there could be both synergism as well as potentiation.

The DBL-1 pathway can act as early as 10 hours or 2 cell divisions before expression of CAT-2::GFP becomes apparent. During the interval, a cascade of events initiated by reception of the signal might occur. Alternatively, a cell memory of receptor signaling, for example, phosphorylation of SMAD proteins, might persist and result in later action at the time of *cat-2* gene expression.

We provide evidence that the DBL-1 signal is partly permissive and partly instructive in patterning the DA fate among ray neurons. It is permissive in that it triggers a prepattern already strongly biased to expression of DA in the correct neurons. However, it is also instructive in the sense that some of the genetic information for the pattern of DA expression is to be found in the *dbl-1* promoter. Evidence for this is provided by the observation that if DBL-1 is supplied from a heat-shock promoter, the pattern of DA neurons is altered. Wild-type regulation of *dbl-1* necessary for generation of normal DA expression could involve timing of ligand expression, the level of ligand, or the spatial distribution of ligand. Our temperature-shift experiments with the temperature-sensitive DAF-4 receptor mutant demonstrate normal DA neuron patterning can result from either early or late action of the signal. This suggests that timing of the signal may not be critical. Our heat-shock experiment shows that the level of ligand expression is important. However, a role for spatial regulation is also suggested, because even low levels of ubiquitous ligand expression resulted in some ectopic DA neurons. The unique positions of the precursor cells of rays 5, 7 and 9 dorsal of the seam could account for their differential exposure to the ligand (Fig. 1D). Further studies are required to determine whether DBL-1 is preferentially expressed from a dorsal source, or, alternatively, whether there might be an inhibitor generated from a ventral source.

The combined actions of the *Hox* gene *egl-5* and the DBL-1 pathway signal in patterning the expression of DA among ray neurons bears many similarities to the combined actions of the *Hox* gene *lin-39* and the EGF-like LIN-3 pathway signal in specification of vulval fates among ventral hypodermal cells (Clark et al., 1993; Clandidin et al., 1997; Maloof and Kenyon, 1998). In both pathways, *Hox* genes act early to initiate pathways of development and late to define specific characteristics of differentiated cells. In both pathways, regulated expression of the ligand plays a role in specifying the pattern, and the *Hox* gene establishes competence. An apparent difference is that vulval development absolutely requires the LIN-3 signal, whereas DA is expressed at significant frequency in the absence of DBL-1. However, in many mutant backgrounds in *C. elegans*, vulval fates are expressed in a signal-independent manner, and in other nematode species vulval development has evolved to become partially or wholly signal-independent (Sommer and Sternberg, 1994; Sommer, 1997; Felix and Sternberg, 1997). Therefore in both tissues the *Hox* gene itself can be sufficient to dictate the differentiated cell state. The role of the signal appears to be to promote and direct this fate to particular cells.

A second apparent difference from vulval development is the absence or lesser role of lateral inhibition in the ray patterning pathway. Interactions between vulval cells establish the distribution of cell fates (Greenwald, 1997). Laser ablation studies did not reveal such interactions among rays or ray progenitor cells (Chow and Emmons, 1994). However, our data here suggest the possibility of lateral inhibition, to explain why both increased and decreased ligand appear to promote the DA fate in some rays. A putative secondary signal, induced by the DBL-1 pathway signal, might promote a refractory state in which the ray cells are insensitive to ligand, as mentioned above.

The relationship between ray neurotransmitter identity and ray morphological identity

The DBL-1 pathway specifies at least two aspects of ray identity, ray morphology and ray neurotransmitter. The unique dorsal location and DA neurotransmitter expression by rays 5, 7 and 9 may be required for the special role of these rays in mating (Liu and Sternberg, 1995). In DBL-1-pathway loss-of-function mutants, rays 5, 7 and 9 appear to take on the morphological identities of their anterior neighbors, fusing with them (Baird et al., 1991; Savage et al., 1996; Krishna et al., 1999; Morita et al., 1999; Suzuki et al., 1999). Fusion presumably results from misexpression or loss of expression of genes involved in cell-cell recognition events, some of which appear to be expressed by the structural cell (Zhang and Emmons, 1995). Therefore, in addition to *cat-2*, the DBL-1 pathway regulates at least one additional class of ray-specific genes expressed by a different branch of the ray sublineage.

Our results indicate that ray morphology and neurotransmitter are specified independently by the DBL-1 pathway, which therefore must act at multiple steps. If action of the DBL-1 pathway at a single step simultaneously defined both the morphological and neurotransmitter identity of a ray, then ray morphology and neurotransmitter would always be correctly associated. However, we found that loss-of-function mutations in the DBL-1 pathway or ectopic expression of DBL-1 ligand could uncouple this association. In numerous instances, a ray with unambiguous morphology typical of a particular ray inappropriately expressed or failed to express DA. For example, in the DBL-1 heat-shock experiments, in 18 instances, ray 4 maintained correct morphology (opening on

the ventral surface of the fan) yet expressed CAT-2::GFP. In 11 instances, it assumed the morphology of ray 5 (opening on the dorsal surface of the fan) yet failed to express CAT-2::GFP. Therefore the DBL-1 signal can transform the morphological phenotype and neurotransmitter phenotype of ray 4 independently to those of ray 5. Fig. 2F shows a converse example, where ray 5 has maintained is dorsal morphology yet has failed to express CAT-2::GFP. Since we show that action of DBL-1 can occur, both to affect morphology and neurotransmitter, after the time when the lineage branches leading to the A-type neuron and the structural cell have separated (Figs 4, 5; data not shown), this supports a conclusion that DBL-1 can act separately to determine neurotransmitter and ray morphology. Having both characteristics determined by a single signaling pathway may help to ensure their coordinate expression.

Conserved mechanisms of catecholaminergic neurotransmitter specification

In vertebrates, signals that direct establishment of TH expression and catecholaminergic phenotype during development have begun to be identified. These studies suggest that specification of the catecholaminergic phenotype in vertebrates and nematodes may share common mechanisms. In the CNS, development of midbrain dopaminergic neurons occurs in the ventral neural tube at axial locations where Sonic Hedgehog and FGF-8 expression overlap (Hynes et al., 1995, Ye et al., 1998). There is no evidence that TGFβ family ligands are involved in specification, but members of this family have been shown to effect survival of dopaminergic neurons (Poulson et al., 1994; Jordon et al., 1997; Reiriz et al., 1999). This neurotrophic role contrasts with our observations that continued DBL-1 signaling is not required for survival of ray dopaminergic neurons or continued expression of their dopaminergic phenotype. Possible developmental roles for BMPs in establishment of TH expression in the vertebrate CNS are suggested, however, by the observation that multiple BMPs are present in developing midbrain floor, where dopaminergic neurons differentiate (Jordon et al., 1997). Activin or BMP-2, in combination with FGF-2 or FGF-4, can induce TH expression in basal forebrain ventricular zone progenitors and their postmitotic progeny in culture (Daadi et al., 1998).

In the vertebrate PNS, BMPs have been implicated in the acquisition of adrenergic phenotype in developing postganglionic sympathetic neurons. The role of BMPs in this developmental process may be similar to that of DBL-signaling in the specification dopaminergic phenotype among ray neurons. First, in both systems, ectopic expression of the ligand can induce TH expression in a larger population of cells than observed in wild type (Varley and Maxwell, 1996; Reissmann et al., 1996; Varley et al., 1998). Second, additional prepatterning steps make cells competent to respond to the signal. In the developing sympathetic nervous system, notochord and other axial structures provide essential signals that may set up such competence (Teillet and Le Douarin, 1983; Stern et al., 1991; Groves et al., 1995; Lo et al., 1999). Further molecular and genetic studies should reveal whether the parallels between ray dopaminergic development and vertebrate catecholaminergic neuron development reflect conserved mechanisms.

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