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The *C. elegans* POU-domain transcription factor UNC-86 regulates the *tph-1* tryptophan hydroxylase gene and neurite outgrowth in specific serotonergic neurons

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SUMMARY

A fundamental question in developmental neurobiology is how a common neurotransmitter is specified in different neuronal types?. We describe cell-specific regulation of the serotonergic phenotype by the *C. elegans* POU-transcription factor UNC-86. We show that *unc-86* regulates particular aspects of the terminal neuronal identity in four classes of serotonergic neurons, but that the development of the ADF serotonergic neurons is regulated by an UNC-86-independent program. In the NSM neurons, the role of *unc-86* is confined in late differentiation; the neurons are generated but do not express genes necessary for serotonergic neurotransmission. *unc-86*-null mutations affect the expression in NSM of *tph-1*, which encodes the serotonin synthetic enzyme tryptophan hydroxylase, and *cat-1*, which encodes a vesicular transporter that loads serotonin into synaptic vesicles, suggesting that *unc-86* coordinately regulates serotonin synthesis and packaging. However, *unc-86*-null mutations do not impair the ability of NSM to reuptake serotonin released from the ADF serotonergic chemosensory neurons and this serotonin reuptake is sensitive to the serotonin reuptake block drugs imipramine and fluoxetine, demonstrating that serotonin synthesis and reuptake is regulated by distinct factors. The NSM neurons in *unc-86*-null mutants also display abnormal neurite outgrowth, suggesting a role of *unc-86* in regulating this process as well.

Key words: *C. elegans*, POU domain, Transcription, Neuron identity, Neurotransmitter, Serotonin, Serotonin reuptake inhibitors, Axon guidance

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter that regulates mood, aggression, satiety and body temperature (for a review, see Lucki, 1998). Drugs that modulate serotonin signaling are potent antidepressants and affect rates of metabolism (Gray et al., 1992; Kaye et al., 1998; Leibowitz and Alexander, 1998). In both vertebrates and invertebrates, serotonergic neurons represent a small population of neurons with diverse morphology and function. The specification of serotonergic phenotypes appears to be a terminal differentiation event – in general, serotonin production starts about 24 hours or later after the neurons exit from the cell cycle (Lidov and Molliver, 1982; Marois and Carew, 1997; Lundell and Hirsh, 1994). Some components of neuronal identity must be shared by all classes of serotonergic neurons, such as enzymes that synthesize serotonin, transporters that load serotonin into synaptic vesicles and that reuptake extracellular serotonin. Others may be restricted to a particular class of serotonergic neuron, such as receptors to specific synaptic inputs and factors mediating axon pathfinding. Factors required for early stages of the development of serotonergic neurons are beginning to be elucidated (Matise et al., 1998; Ye et al., 1998; Van Doorninck et al., 1999; Briscoe et al., 1999; Hynes and Rosenthal, 1999). An intriguing issue is whether there are 'master' genes that switch on serotonergic phenotypes in all neuronal classes or whether different regulatory genes for each neuronal type coordinately regulate transmitter and other aspects of the final neuronal identity.

The nematode *C. elegans* has a relatively simple serotonergic system. Of 302 neurons in an adult hermaphrodite, nine neurons of five distinct classes are detected with antibodies raised against serotonin (Horvitz et al., 1982; Rand and Nonet, 1997). These neurons are generated from different lineages during embryogenesis (Sulston et al., 1983). Serotonin immunoreactivity can be detected in the ADF, NSM, AIM and RIH neurons shortly after hatching, and in the HSN neurons only in adults (Desai et al., 1988; Sze et al., 2000).

Biosynthesis of serotonin in *C. elegans* requires the *tph-1* gene, which encodes a tryptophan hydroxylase that catalyzes the first and rate-limiting step of serotonin biosynthesis (Sze et al., 2000). *tph-1* is expressed in the serotonergic neurons, and

animals bearing a *tph-1* deletion mutation show no detectable serotonin immunoreactivity (Sze et al., 2000). The *cat-1* gene encodes a vesicular monoamine transporter that loads serotonin into synaptic vesicles and is important for serotonin signaling (Duerr et al., 1999; Nurrish et al., 1999).

The POU-domain transcription factor UNC-86 is expressed in 57 neurons throughout the life of the animal, including the NSM, AIM, RIH and HSN serotonergic neurons (Finney et al., 1988; Finney and Ruvkun, 1990). A transcriptional cascade downstream of UNC-86 in the touch receptor neurons has been characterized: UNC-86 continuously regulates the expression of the LIM-homeobox gene *mec-3* (Way and Chalfie, 1989; Xue et al., 1992), and UNC-86-MEC-3 complex regulates touch receptor specific components, such as the MEC-7 β-tubulin and MEC-4 ion-channel (Duggan et al., 1998), to specify and maintain mechanosensory function. These studies indicate that UNC-86 may interact with cell-specific co-factors to regulate specific neuronal components. The mammalian *unc-86* orthologs, *Brn3a*/Pou4f1 and *Brn3b/Pou4f2,* also mediate development, maintenance and function of the endocrine and nervous systems (for a review, see McEvilly and Rosenfeld, 1999) and regulate the expression of axon guidance genes (Lakin et al., 1995; Smith et al., 1997a; Erkman et al., 2000). Although it has been shown that *unc-86* is required for the maturation of the HSN neurons (Desai et al., 1988), the role of *unc-86* in determining the serotonergic neural fate has not been analyzed.

There are several examples that homeodomain transcription factors act in postmitotic cells to specify neurotransmitter phenotypes in particular neurons. In *C. elegans*, UNC-30 directly regulates the expression of genes encoding GABA synthetic enzyme glutamate decarboxylase and vesicular GABA transporter in just one class of the neurons (Eastman et al., 1999), whereas GABAergic phenotype in the other neurons requires the LIM-factor LIM-6 (Hobert et al., 1999). Similarly, in *Drosophila*, *islet* acts in subsets of ventral cord neurons to specify the serotonergic and dopaminergic neural fates (Thor and Thomas, 1997), and the POU-domain factor *Cfla* regulates the dopamine decarboxylase gene in specific dopaminergic neurons (Johnson and Hirsh, 1990).

We describe the effect of *unc-86*-null mutations on serotonin synthesis, packaging and reuptake in a subset of the serotonergic neurons. The results from this study support the notion that serotonergic phenotypes in different neurons may be regulated by different transcription factors, and within the same neuron serotonin biosynthesis, packaging, reuptake, and other properties are regulated by distinct regulatory programs.

MATERIALS AND METHODS

Worm strains

The strains used in this study were wild-type *C. elegans* Bristol strain (N2), *unc-86(n846)*, *unc-86(e1416)*, *tph-1(mg280)* and *nss-1(yz12)*.

Constructs

tph-1-ABCDE::gfp is a translation fusion of the *tph-1* genomic sequence encompassing 3.1 kb 5′-upstream sequence from the translation start to the beginning of exon 4 and GFP in pPD95.75 (Sze et al., 2000). *tph-1-BCDE::gfp*, *tph-1-CDE::gfp*, *tph-1-DE::gfp* and *tph-1-E::gfp* are PCR fragments amplified using *tph-1-ABCDE::gfp* as a template. The forward PCR primers correspond to the particular region of the *tph-1* 5′-upstream sequence as indicated in Fig. 4, and the reverse primers correspond to the sequence downstream of the *unc-54* 3′-uncoding sequence on pPD95.75 (A. Fire). Deletion of the region D in *tph-1-BCE::gfp* was generated by fusing the fragments corresponding to the region BC and *tph-1-E::gfp*, using PCR with primers containing the joint site sequence. *gtpch-1::gfp* is a translation fusion of a predicted GTP-cyclohydrolase 1 (*gtpch-1*) and GFP. The sequence of *gtpch-1* is the cosmid F32G8.6, and the structure of GTP-cyclohydrolase was predicted by Genefinder (*C. elegans* Sequencing Consortium, 1998). The *gtpch-1* sequence including 2 kb upstream from the predicted translation start to amino acid 63 of predicted *gtpch-1* in exon 2 was amplified from N2 genomic DNA, using PCR. The amplified *gtpch-1* fragment was fused to GFP and *unc-54* 3′-uncoding sequence in pPD95.75, by PCR with primers containing the joint site sequence. The PCR amplified fusion products were directly used to generate transgenic animals.

GFP expression

The expression of GFP was observed in transgenic animals carrying individual GFP fusion gene constructs. The transgenic animals were generated by microinjection of the GFP fusion genes into wild-type animals, with the plasmid pRF4 that contains the dominant Rol-6 gene co-injected as a genetic marker for the transgene (Mello et al., 1991). The plasmid containing *tph-1-ABCDE::gfp* construct was injected at 50 ng μl^{-1}, the PCR products of GFP fusion were injected without dilution, and pRF4 was injected at 100 ng μl^{-1}. The *tph-1-ABCDE::gfp* transgene was integrated to wild-type chromosome (Sze et al., 2000); four independent integrated lines were obtained. All other GFP reporters were transmitted as extrachromosomal arrays. For each construct, two to four transgenic lines were examined. To determine the effect of *unc-86* and *tph-1* on the GFP expression, two lines of each GFP construct were crossed into *unc-86(n846)*, *unc-86(e1416)* and *tph-1(mg280)*.

Immunoanalysis

The serotonin antibody staining was performed using a whole-mount procedure as described (McIntire et al., 1992; Sze et al., 2000). For imipramine and fluoxetine experiments, the drugs were dissolved in distilled water and the solution was poured onto NG agar plates with a lawn of *E. coli OP50*. After the drug plates were dried for 2 hours, worms were washed off from their culture plates and transferred onto the drug plates, incubated at 20°C for 12-16 hours, then fixed for the antibody staining. Initially, we incubated worms on the plates containing imipramine at the final concentration of 0.75 mg ml^{-1}, and fluoxetine at 1 mg ml^{-1} (Weinshenker et al., 1995). We found that worms did not move much and tended to group together, thus they may not be able to access the drugs fully. Therefore, we reduced imipramine to 0.4 mg ml^{-1} and fluoxetine to 0.5 mg ml^{-1}. The control animals were raised in parallel but without drug treatments. The staining was carried out many times; every time animals treated with or without drugs were stained in parallel. The summary of two most recent trials is reported here (Table 1; Fig. 3).

Isolation of *nss-1(yz12)* and construction of *nss-1(yz12); unc-86*

nss-1(yz12) was isolated from a genetic screen for genes that regulate *tph-1* expression in specific serotonergic neurons. We mutagenized wild-type hermaphrodites carrying integrated *tph-1-ABCDE::gfp* with ethyl methanesulfonate, and F_2 progeny that failed to express GFP were isolated. *nss-1(yz12)* and 23 other mutants were identified based on the reduction/absence of GFP in the ADF chemosensory neurons from screening of about 6500 haploid genomes. None of the mutants showed 100% absence of GFP expression in ADF, but *nss-1(yz12)* and five other mutants showed relatively high penetrance. *nss-1(yz12)* mutants have been backcrossed four times. About 65-90% of the backcrossed *yz12* animals showed no GFP or weak GFP in ADF, whereas the GFP expression in NSM and HSN was equivalent to that

in wild-type (n>200). The *nss-1(yz12)* mutation is recessive and mapped to the X chromosome. Double mutants of *unc-86(n846)* or *unc-86(e1416)* and *nss-1(yz12)* were constructed by following the Mec phenotype of *unc-86* (Chalfie and Au, 1989) and the lack of *tph-1::gfp* expression in ADF of *nss-1(yz12)*. In each case, the strain was confirmed by outcrossing the double mutants and reisolating the single mutations.

RESULTS

unc-86 regulates *tph-1* expression in a subset of the serotonergic neurons

Of five classes of the neurons that accumulate serotonin, four classes express the transcription factor UNC-86: the NSM neurons, a pair of pharyngeal secretory neurons that modulate feeding behavior; the HSN neurons, a pair of motoneurons that mediate egg-laying behavior; and a single RIH and a pair of the AIM interneurons in the head region (Avery and Horvitz, 1989; Desai et al., 1988; White et al., 1986). UNC-86 expression begins in the precursor of the NSM neurons, but in AIM, RIH and HSN, UNC-86 expression is initiated in the postmitotic differentiating neurons. In all these neurons, UNC-86 expression is maintained throughout the life of the animal (Finney and Ruvkun, 1990).

UNC-86 is not required for the generation of these serotonergic neurons. For example, the NSM, AIM and RIH neurons are generated and have no obvious morphological defects in *unc-86*-null mutants (Finney and Ruvkun, 1990). In an *unc-86*-null mutant, the HSN neurons are generated but do not acquire adult HSN features (Desai et al., 1988).

The *tph-1* gene encodes a tryptophan hydroxylase, which is required for serotonin biosynthesis (Sze et al., 2000). This gene is expressed in the serotonergic neurons as they begin to accumulate serotonin (Sze et al., 2000). To assess whether *unc-86* specifies the serotonergic phenotype in these neurons, we used a GFP fusion to *tph-1* to examine the effect of *unc-86*-null mutations on *tph-1*expression. A *tph-1::gfp* fusion gene is expressed in ADF, NSM, HSN and very weakly in RIH and AIM (Sze et al., 2000) (Fig. 1). We crossed the *tph-1::gfp* fusion gene into two *unc-86*-null alleles, *n846* and *e1416*. In both *unc-86*-null alleles, GFP expression was only observed in the pair of the ADF neurons (Fig. 1), which do not express *unc-86*. To ensure that the lack of GFP expression was not due to a defect in the fusion gene, we examined GFP expression from two independent *tph-1::gfp* integrant lines and from extrachromosomal arrays. No GFP was observed in *unc-86*-expressing cells at any developmental stage of *unc-86*-null mutants. These observations suggest that UNC-86 is specifically required for *tph-1* expression in *unc-86*-expressing neurons.

unc-86-null mutations also affect the expression of the CAT-1 serotonin vesicular transporter

The *C. elegans* homeobox gene *unc-30* coordinately regulates the expression of the GABA synthetic enzyme glutamate decarboxylase and GABA vesicular transporter (Eastman et al., 1999). In both vertebrates and invertebrates, the genes encoding choline acetyltransferase and acetylcholine vesicular transporter are co-regulated by a common promoter (Rand, 1989; Eiden, 1998; Kitamoto et al., 1998). To determine whether genes for serotonin synthesis and packaging also are coordinated regulated, we examined the effect of *unc-86*-null mutations on the expression of the *cat-1* vesicular monoamine transporter gene, which transports serotonin, dopamine and perhaps other monoamine neurotransmitters (Duerr et al., 1999; Nurrish et al., 1999).

cat-1::gfp is a functional fusion of GFP to the entire protein coding segment of the *cat-1* gene and is integrated onto a wild-type

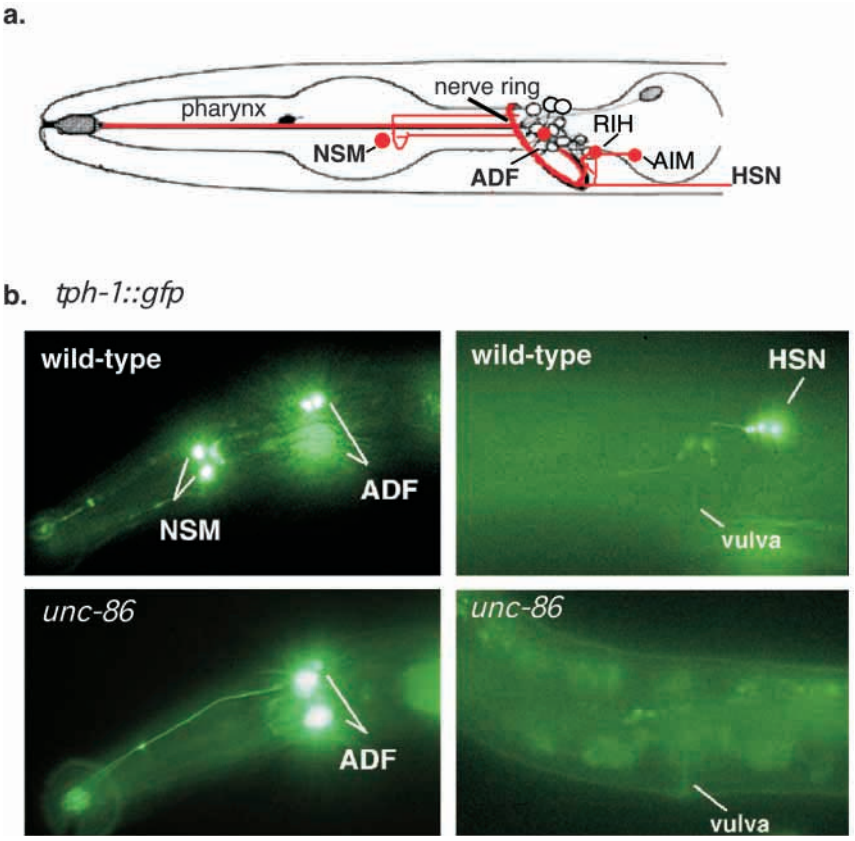

Fig. 1. Effects of *unc-86*-null mutations on the expression of *tph-1::gfp*. (A) The position of serotonergic neurons in the head and the axon from HSN (shown in red). The drawing is adapted from Starich et al. (Starich et al., 1995) and White et al. (White et al., 1986). (B) GFP expression of the integrated *tph-1-ABCDE::gfp* fusion gene in wild-type and *unc-86*-null adult hermaphrodites. Anterior is towards the left. In wild type, GFP was predominantly expressed in ADF, NSM and HSN. In *unc-86*-null mutants, GFP expression was observed in ADF, but not in NSM and HSN at any developmental stage. The UNC-86 protein is expressed in NSM and HSN, but not in ADF (Finney and Ruvkun, 1990). Two *unc-86*-null alleles, *n846* and *e1416* exhibited the same expression pattern; *unc-86(n846)* animals are shown. Note that the GFP fusion to the *tph-1* sequence has punctate expression pattern, as it appears as two puncta in the ADF neuron.

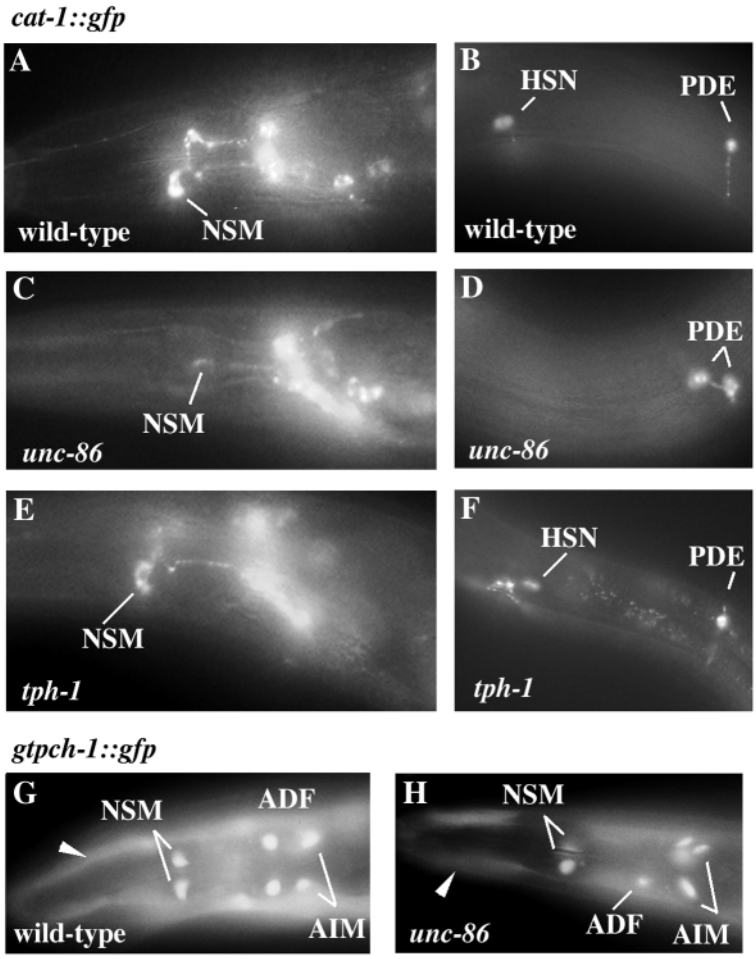

Fig. 2. *unc-86* affects specific genes in the serotonergic neurons. (A-F) Effects of *unc-86* on the expression of the *cat-1* monoamine vesicular transporter. Expression of an integrated *cat-1::gfp* fusion gene was examined in wild type, *unc-86*-null and *tph-1* mutants. (A,B) In wild-type, this functional *cat-1* fusion to GFP was specifically expressed in all predicted serotonergic and dopaminergic neurons; representative expression in the serotonergic NSM, HSN, dopaminergic PDE neurons and the nerve ring is shown. (C,D) In *unc-86* mutants, GFP expression in HSN was completely absent, and in NSM was significantly reduced. However, unlike *tph-1::gfp* (Fig. 1), *cat-1::gfp* expression in NSM was not completely absent (C). Note the *unc-86* mutants had extra PDE neurons (D) (Finney and Ruvkun, 1990) expressing *cat-1::gfp*. (E,F) In the serotonin synthesis mutant *tph-1(mg280)*, *cat-1::gfp* levels in NSM and other neurons were indistinguishable from wild-type. (G,H) Expression of a GFP fusion to the F32G8.6 GTP-cyclohydrolase 1, *gtpch-1::gfp*, in wild-type (G) and *unc-86* mutant (H) animals. In wild-type animals, GFP was observed in all predicted serotonergic and dopaminergic neurons, as well as in the head and body wall muscles (arrowhead). Except for the absence of GFP in HSN (arrowhead in H), the expression levels in *unc-86* mutants were very similar to wild type. All animals shown are adults, and the anterior is to the left.

chromosome (S. Nurrish, personal communication). Fig. 2A,B show that *cat-1::gfp* is specifically expressed in the serotonergic and dopaminergic neurons (Sulston et al., 1975; Rand and Nonet, 1997). We crossed the *cat-1::gfp* fusion gene into *unc-86(n846)* and *unc-86(e1416)* animals, and found that GFP fluorescence was absent in AIM, RIH and HSN, significantly reduced in NSM, whereas the expression levels in other neurons were not changed (Fig. 2C,D; not shown). Thus, UNC-86 activates both *tph-1* and *cat-1* expression in the NSM, AIM, RIH and HSN neurons. Unlike *tph-1::gfp*, *cat-1::gfp* expression in NSM is not completely absent in the *unc-86*-null mutants, suggesting that factors in addition to UNC-86 contribute to *cat-1* expression in NSM.

Since *unc-86*-null mutations affect *tph-1* expression, the reduced *cat-1* expression observed in NSM, AIM, RIH and HSN might result from the lack of serotonin production rather than the lack of *unc-86*. However, no change in *cat-1::gfp* expression was observed in *tph-1(mg280)* mutants that contain no detectable serotonin (Fig. 2E,F). Thus, UNC-86 activates *cat-1::gfp* expression in the NSM, AIM, RIH and HSN neurons, but has no effect on *cat-1* expression in the dopaminergic neurons or the ADF serotonergic neurons that do not express UNC-86.

UNC-86 is not required for the survival or gross fate-specification of the NSM, AIM and RIH serotonergic neurons

The neurons do not express *tph-1*, and *cat-1* in the *unc-86*-null mutant may require *unc-86* to acquire their specific serotonergic neuronal fate and/or for survival. In *unc-86*-null mutants, *tph-1::gfp* was not observed at any developmental stage, and there was no obvious difference in the expression level of *cat-1::gfp* between the mutant larvae and adults. Thus, the loss of *tph-1* and *cat-1* expression is not due to progressive neuronal degeneration. To assess whether these neurons express other markers of serotonergic neural fates, and thus rule out homeotic transformation of the neural fate, we have examined the effect of *unc-86*-null mutations on the expression of another gene specific for catecholaminergic neurons. The gene F32G8.6 encodes a probable GTP-cyclohydrolase I (GTPCH-1), a co-factor of tryptophan hydroxylase (Sanders-Bush and Mayer, 1996). We constructed a *gtpch-1::gfp* fusion gene, and examined the expression pattern in transgenic animals. In wild-type animals, GFP was observed in all identified serotonergic and dopaminergic neurons and many muscle cells (Fig. 2G). We crossed two independent *gtpch-1::gfp* transgenic arrays into *unc-86*-null mutants. Although GFP expression was not observed in HSN, the expression levels in NSM and the other serotonergic neurons were not changed in the *unc-86*-null mutants (Fig. 2H). All of the serotonergic neurons except HSN exhibited normal position, size and shape, indicating these neurons were generated and survived in the *unc-86* mutant. These observations indicate that *unc-86*-null mutations do not alter the overall fate of NSM, AIM and RIH, only their expression of specific genes. In the case of HSN, UNC-86 appears to act higher in the cascade of neural differentiation, upstream of *tph-1*, *cat-1* and *gtpch-1*.

Serotonin-reuptake and serotonin-synthesis are independent events in NSM development

The NSM and ADF neurons continue to accumulate serotonin in *unc-86(n846)* and *unc-86(e1416)* null-mutant animals, as

revealed by immunofluorescent detection of serotonin with anti-serotonin antibodies (Fig. 3C; Table 1). Because *tph-1* is essential for serotonin synthesis (Sze et al., 2000) and *unc-86*-null mutants have no detectable *tph-1::gfp* expression in NSM (Fig. 1), we thought of two possible sources of serotonin in NSM of *unc-86* mutants: (1) the NSM neurons take up extracellular serotonin; (2) another transcription factor also activates *tph-1* expression but its target is not included in our GFP reporter construct.

To assess the role of extracellular serotonin transport, we examined NSM serotonin in *unc-86* mutants treated with the serotonin reuptake inhibitors imipramine or fluoxetine (Baldessarini, 1996). Imipramine stimulates *C. elegans* egg laying and pharyngeal pumping in wild-type but not in serotonin-deficient mutants (Weinshenker et al., 1995; Avery and Horvitz, 1990). Thus, imipramine functions in *C. elegans* presumably by enhancing serotonin signaling by preventing reuptake of serotonin from the synapse. We found that imipramine treatment significantly reduced serotonin in the NSM but not in the ADF neurons of *unc-86* mutants (Fig. 3E; Table 1): 74-86% of the NSM neurons had no detectable serotonin and 14-20% showed weak serotonin in the imipramine treated *unc-86* mutant animals, whereas 100% of NSM accumulated serotonin in untreated *unc-86* mutant animals. No change in ADF serotonin level was observed (Fig. 3E,F), indicating that the imipramine treatment did not reduce serotonin accumulation in general. No reduction of NSM serotonin immunofluorescence was observed in wild-type animals treated with imipramine (Fig. 3F). Similarly, we observed reduced serotonin in NSM of *unc-86* mutants treated with fluoxetine (Table 1): 36% had no detectable serotonin and 54% showed weak serotonin level in NSM. These results indicate that UNC-86 activates serotonin synthesis in all serotonergic neurons except ADF, but that the NSM neurons continue to take up serotonin, presumably released from ADF, in an *unc-86* mutant. We do not exclude the possibility that additional transcriptional regulators can activate low levels of serotonin synthesis in the absence of *unc-86*.

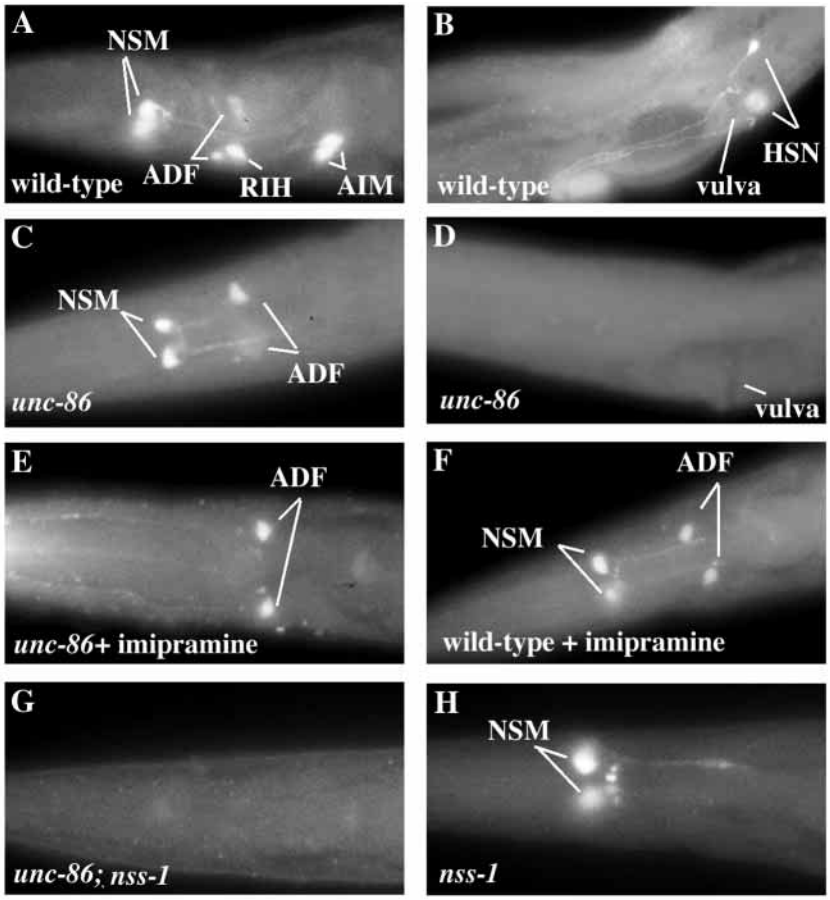

Fig. 3. Analysis of NSM serotonin immunoreactivity in adult hermaphrodites. (A,B) In wild-type animals, nine neurons of five classes were stained by the anti-serotonin antibody. In general, the frequency of the AIM and RIH neurons stained is lower than of NSM, ADF and HSN, and the intensity of the staining in AIM and RIH is often lower. (C,D) In *unc-86*-null mutants, serotonin immunoreactivity in NSM and ADF remained, unlike in AIM, RIH and HSN. The ADF neurons are the only class of the serotonergic neurons that does not express *unc-86*, and *unc-86* mutations had no effect on ADF serotonin immunoreactivity. (E,F) Treatment of animals with the serotonin reuptake block drug imipramine significantly reduced NSM but not ADF serotonin immunoreactivity in *unc-86* mutants (E), and this effect was not observed in wild-type animals treated with imipramine at the same concentration (F). (G) No serotonin could be detected in the *unc-86(e1416);nss-1(yz12)* double mutant. *unc-86(n846)*; *nss-1(yz12)* showed similar staining pattern (not shown). (H) The *nss-1(yz12)* mutation specifically affects serotonin immunoreactivity in ADF. Adult animals are shown, and the anterior is towards the left. For each strain, the immunostaining was performed many times, and quantification of the observation from two recent trials is presented in Table 1.

NSM and ADF are the only neurons that accumulate serotonin in *unc-86* hermaphrodites (Fig. 3C,D). To determine if ADF is the source of NSM serotonin in *unc-86* mutants, we constructed double mutants of *unc-86* and a mutation affecting serotonin biosynthesis in ADF. We have isolated several *nss* (neuron-specific serotonin defective) mutations that affect serotonergic phenotypes in specific serotonergic neurons (see Materials and Methods). The *nss-1(yz12)* mutation specifically affects serotonin accumulation in ADF: 72% of *nss-1(yz12)* mutants showed no ADF serotonin immunofluorescence, whereas the NSM immunofluorescence was equivalent to that in wild-type (Fig. 3H; Table 1). We constructed *unc-86* and *nss-1(yz12)* double mutant animals, and found that *nss-1(yz12)* affects NSM serotonin accumulation in the *unc-86* mutant background (Fig. 3G; Table 1): 56% of the double mutants had no detectable serotonin immunofluorescence, whereas the remaining animals had low serotonin immunofluorescence in ADF, but NSM serotonin immunofluorescence was either completely absent or very faint, only 15 of out 188 animals examined showed relatively strong NSM serotonin immunofluorescence. As *nss-1(yz12)* does not completely eliminate ADF serotonin, this may account for the residual NSM serotonin in the double mutants. Thus, NSM can take up serotonin from ADF and *unc-86*-null mutations do not disrupt

Table 1. Serotonin immunoreactivity in *unc-86*-null mutants

		% of ADF			% of NSM						
Mutant	Drug	Strong	Weak	None	Strong	Weak	None	HSN	AIM	RIH	*n*
N2	None	100	0	0	100	0	0	100	94	69	67
	Imipramine	100	0	0	100	0	0	100	na	na	211
	Fluoxetine	100	0	0	100	0	0	na	na	na	82
unc-86(n846)	None	100	0	0	100	0	0	0	1	0	152
	Imipramine	100	0	0	6	20	74	0	na	na	277
unc-86(e1416)	None	100	0	0	100	0	0	0	0	0	132
	Imipramine	100	0	0	0.3	14	86	0	na	na	284
	Fluoxetine	100	0	0	10	54	36	na	na	na	78
nss-1(yz12)	Imipramine	12	16	72	100	0	0	100	na	na	122
nss-1; umc-86(e1416)	None	30	14	56	8	19	72	0	na	na	188

The worms were stained with anti-serotonin antibody using a whole-mount procedure (McIntire et al., 1992). Imipramine concentration was 0.4 mg ml^{-1} and fluoxetine concentration was 0.5 mg ml^{-1} (see Materials and Methods). The definition of the strength of the immunoreactivity: Strong, equivalent to that shown in Fig. 3A,B; weak, the entire range from less than strong to very faint; none, not visually detectable. For each strain, percentage of animals in each category is shown. *n* is the number of animals examined, which represents two independent staining trials. Mixed staged animals were stained. HSN immunoreactivity was scored only in adults; other neurons were scored in larvae and adults. na, not analyzed.

the serotonin reuptake machinery in NSM. These results suggest that the components for serotonin production and reuptake are regulated by distinct regulatory programs in NSM development.

We did not observe serotonin immunofluorescence in AIM, RIH and HSN in *unc-86* mutants, even without serotonin reuptake inhibitor treatment. The absence of serotonin in HSN supports previous studies that *unc-86* is required for several features of HSN maturation (Desai et al., 1988). Serotonin levels in AIM and RIH are lower than in HSN, NSM and ADF; thus, we may not be able to detect it if the level is further reduced because of reduced serotonin synthesis in the *unc-86* background. Alternatively, *unc-86* may have a broader role in RIH and AIM differentiation.

tph-1 regulatory elements

To distinguish whether *tph-1* expression in ADF and *unc-86*-expressing cells is mediated by different regulators, or there is a common *tph-1* regulator that may act downstream of *nss-1* and *unc-86*, we constructed GFP fusion genes that bear various *tph-1* regulatory elements and surveyed the expression pattern in transgenic animals. The *tph-1-ABCDE::gfp* fusion gene has GFP fused at exon 4 of *tph-1* and contains 3.1 kb 5′-upstream uncoding sequence, and was strongly expressed in NSM, ADF at all stages and in HSN only at the adult stage (Fig. 4). We have shown that a *tph-1* gene containing this upstream region and the entire coding region with introns can direct wild-type levels of serotonin synthesis in all the serotonergic neurons in a *tph-1* deletion mutant (Sze et al., 2000). We constructed a series of deletions of the 5′-region from *tph-1-ABCDE::gfp* (Fig. 4), introduced individual constructs into wild-type animals, and the expression pattern of each construct in two to four transgenic lines has been examined. The construct *tph-1-BCDE::gfp*, which contains 377 bp upstream from the translation start site, recapitulated the *tph-1-ABCDE::gfp* expression pattern (Fig. 4B). However, deletion to –238 bp (*tph-1-CDE::gfp*) resulted in reduced GFP level in ADF, and a further deletion to –158 (*tph-1-DE::gfp*) greatly diminished GFP expression in ADF, but these deletions had no effect on the expression level in *unc-86*-expressing cells (Fig. 4B). These observations indicate that different transcription factors regulate *tph-1* expression in ADF and *unc-86*-expressing cells. The transcription factors, possibly encoded or regulated by *nss-1*, may activate *tph-1* expression in ADF.

Interestingly, GFP expression of other fusion constructs in the HSN neurons was only observed in the adults, but 93% of the transgenic animals carrying the construct *tph-1-DE::gfp* began to express GFP in HSN at the first larval (L1) stage (*n*=66). The pair of the HSN motoneurons modulates egg-laying behavior, and serotonin is observed only in adult HSN (Desai et al., 1988). Our results suggest that this temporal regulation of serotonin synthesis is mediated by the sequence located between –239 to –159 of the *tph-1* gene.

Deletion to –81 of *tph-1* (*tph-1-E::gfp*) greatly reduced GFP expression in NSM and HSN, as well as in ADF (Fig. 4B). To determine the sequence specifically mediating *tph-1* expression in these UNC-86-expressing cells, we constructed internal deletions. The construct *tph-1-BCE::gfp* is a fusion of the sequence –377 to –166 and *tph-1-E::gfp*. This internal deletion of the sequence between –165 and –82 of *tph-1* significantly reduced GFP levels in NSM and HSN but had no effect on ADF: 37% of the transgenic worms showed no GFP and 62% showed very weak GFP in NSM; 86% showed no GFP and 14% weak GFP in HSN; whereas 100% showed normal GFP levels in ADF (*n*=65). These results suggest that the sequence between –159 and –82 of *tph-1* plays a major role in *tph-1* expression in NSM and HSN.

A sequence motif (GCGCATAATAAAACAAT) at –98 to –82 matches the consensus UNC-86 binding site (Gruber et al., 1997). We used two approaches to test if UNC-86 directly activates *tph-1* expression. First, we deleted this motif sequence from the construct *tph-1-BCDE::gfp*. We observed that the deletion caused a partial reduction of GFP expression in NSM and HSN: 9% showed no GFP and 50% showed weak GFP in NSM, and 8% showed no GFP and 30% showed weak GFP in HSN (*n*=70, from four transgenic lines). For comparison, the non-truncated *tph-1-BCDE::gfp* construct was expressed 100% in NSM, and 97% in HSN (*n*=36). Second, we tested interaction of the UNC-86 protein with the *tph-1* promoter. In the touch receptor neurons, although UNC-86 interacts with MEC-3 to activate the transcription of *mec-3* and *mec-7*, UNC-86 on its own binds to the targets in the promoter of these genes (Xue et al., 1992; Lichtsteiner and Tjian, 1995; Duggan et al., 1998). We tested the binding of

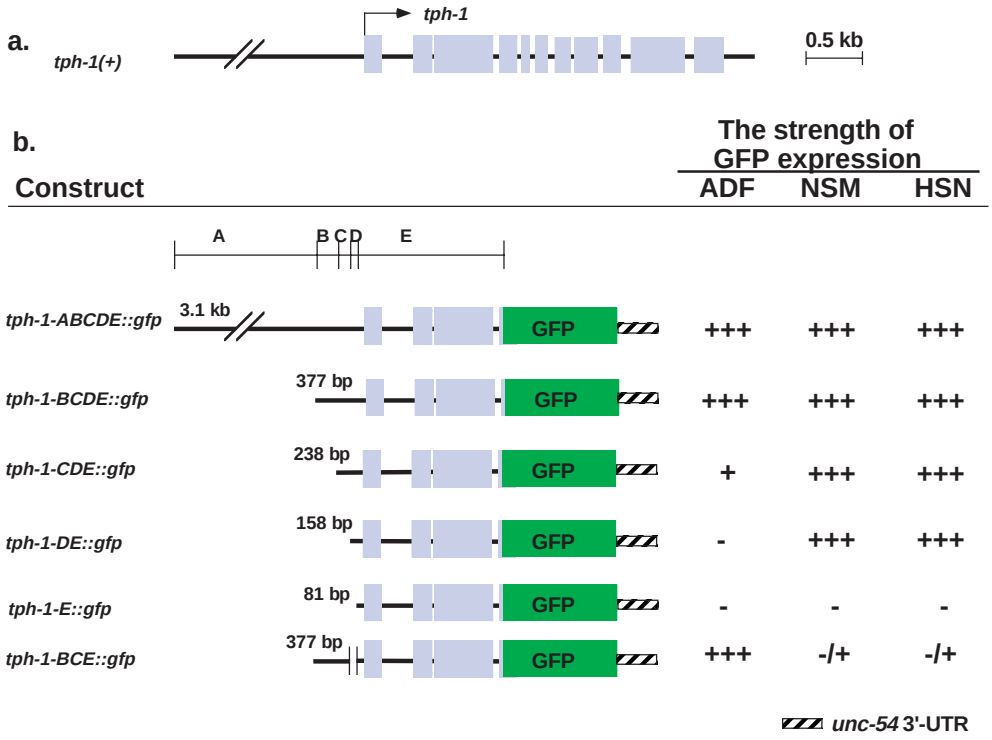

Fig. 4. *tph-1* regulatory elements. The expression of various GFP fusion to *tph-1* in transgenic animals. (A) The genomic structure of the *tph-1* gene. *tph-1* exons are indicated by filled boxes and untranslated regions are indicated as lines. (B) The strength of GFP expression of various constructs. The construct *tph-1-ABCDE::gfp* was integrated onto the chromosome; different lines have the construct integrated onto different chromosomes. The other constructs were transmitted as extrachromosomal arrays. The number of transgenic lines carrying each construct has been examined: *tph-1::ABCDE::gfp*, two lines; *tph-1-BCDE*, four lines; *tph-1-CDE::gfp*, three lines; *tph-1-DE::gfp*, four lines; *tph-1-E::gfp*, three lines; *tph-1-BCE::gfp*, three lines. Because GFP levels in AIM and RIH are too weak to survey reliably, only GFP expression in ADF, NSM and HSN was scored. GFP expression of *tph-1-ABCDE::gfp*, *tph-1-BCDE::gfp* and *tph-1-CDE::gfp* in HSN was observed only in adults, *tph-1-DE::gfp* was expressed in HSN starting at the first larval stage.

the UNC-86 protein synthesized from *E. coli* and in vitro to DNA fragments spanning nucleotides –159 to –4 or –377 to –4 of *tph-1* by gel mobility-shift assays. We observed a weak shifted product that was dependent on UNC-86, and can be competed by unlabelled DNA fragments spanning –159 to –4 of *tph-1*, but not when the consensus UNC-86 binding motif was truncated from this region. However, the binding was much poorer (about 40- to 60-fold less) than the binding of the same protein preparations to the UNC-86 targets CS2 and CS3 on the *mec-3* promoter (data not shown). These results suggest that UNC-86 may interact with a co-factor for high-affinity binding to the *tph-1* promoter, or UNC-86 indirectly regulates *tph-1* expression.

Together, the analyses of the *tph-1* promoter indicate that *tph-1* expression in the ADF and UNC-86-expressing serotonergic neurons is controlled by different regulators, and that distinct regulators mediate the temporal and spatial expression of *tph-1* in the HSN neurons.

NSM exhibit neurite outgrowth defects in *unc-86* mutants

Does *unc-86* specifically regulate *tph-1* and *cat-1* expression, or does it also regulate other aspects of the final identity of the serotonergic neurons? The *unc-86* mammalian orthologs *Brn3a* and *Brn3b* have been shown to regulate axon outgrowth and pathfinding (Lakin et al., 1995; Smith et al., 1997a; Erkman et al., 2000). We examined the effect of *unc-86* on NSM morphology as revealed by serotonin immunofluorescence. The pair of the NSM secretory neurons is bipolar. The cell bodies are located symmetrically in the subventral nerve cord. Both processes extend from the posterior of the cell body: one process runs posteriorly, the other one runs across the ventral side to the dorsal nerve cord, then turns towards posterior, both processes terminate before reaching the terminal bulb of the pharynx (Albertson and Thomson, 1976) (Fig. 5A). *unc-86*-null mutations do not affect the cell position, but defects in neurite outgrowth and pathfinding were observed in some of the mutant animals (Fig. 5B-E): 38% showed extra neurite outgrowth; 18% exhibited one posteriorly directed process; in rare cases, the neurons had one process running anteriorly and the other posteriorly (n=164). Similar defects were observed in both *unc-86(n846)* and *unc-86(e1416)* alleles, and were also revealed in the *unc-86*-mutants carrying *gtpch-1::gfp* (not shown). As *tph-1* mutants that do not produce serotonin show a normal NSM axon outgrowth (Sze et al., 2000), we suggest that these outgrowth defects are not due to decreased serotonin signaling, but that *unc-86* regulates genes involved in axon outgrowth and pathfinding in NSM.

DISCUSSION

Our results showed that *unc-86* regulates serotonergic phenotypes in four classes of the serotonergic neurons. In the NSM neurons *unc-86* regulates the expression of the serotonin synthetic enzyme gene *tph-1*, upregulates the *cat-1* vesicular transporter gene, and controls neurite outgrowth, but is not required for the serotonin reuptake. Similarly in the AIM, RIH and HSN neurons, UNC-86 activates the expression of *tph-1* and *cat-1*. By contrast, the serotonergic phenotype of the ADF neurons is specified by an UNC-86-independent program. These results show that serotonergic phenotype may be specified by different transcription factors in different neurons, and in a given type of neuron, distinct aspects of the serotonergic phenotype – synthesis, packaging,

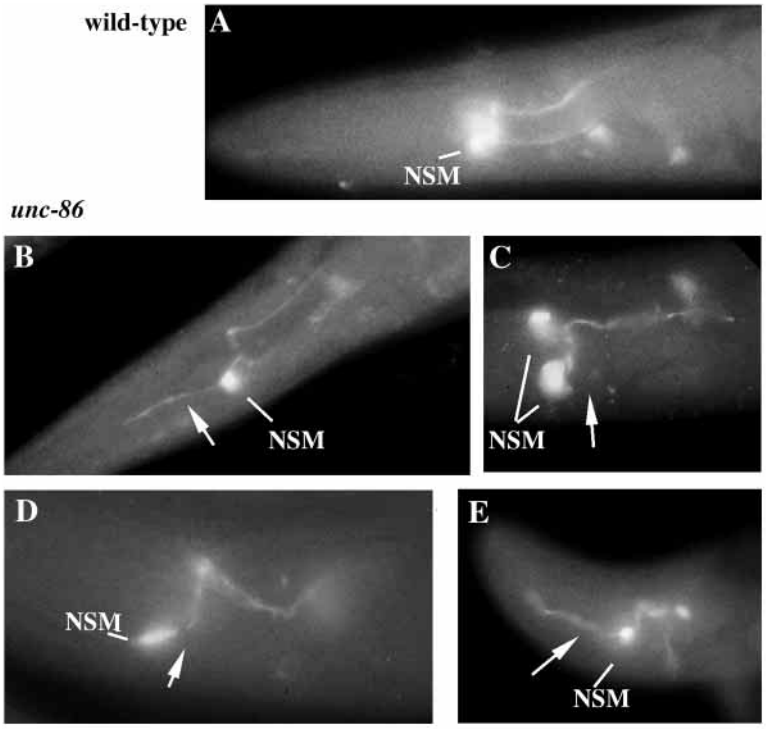

Fig. 5. Analysis of NSM morphology in *unc-86* mutants. Serotonergic neurons in wild-type (A) and *unc-86* mutants (B-E) were visualized by anti-serotonin antibody staining. In wild-type animals (A), NSM has two posterior-directed processes. The position and the gross axon morphology of NSM in *unc-86*-null mutants are indistinguishable from wild-type, but neurite outgrowth defects were observed. Representative examples of outgrowth defects of NSM are shown. The arrows indicate neurite outgrowth defects in the corresponding neurons. In most cases, an extra neurite extended anteriorly to various lengths (B), one of the posteriorly directed axon was either premature terminated (C) or missing (D). In some cases, NSM had one anterior- and one posterior-directed process (E). Similar defects were observed in *unc-86(n846)* and *unc-86(e1416)*, and there was no obvious difference in the frequency of the defects observed in the larvae and adults.

and reuptake – are controlled by different regulatory mechanisms.

unc-86 regulates serotonergic phenotypes in a subset of serotonergic neurons

UNC-86 is expressed in the NSM, AIM, RIH and HSN serotonergic neurons. The analysis of *unc-86*-null mutants indicates that UNC-86 controls the synthesis of serotonin in these neurons. Desai et al. (Desai et al., 1988) showed that *unc-86* mutations affect serotonin accumulation in HSN, and we found UNC-86 is required for HSN to express a GFP reporter of the *tph-1* tryptophan hydroxylase (Fig. 1). Both serotonin and *tph-1::gfp* expression levels in AIM and RIH are low in wild-type animals, but we could not detect any serotonin immunoreactivity or *tph-1::gfp* expression in AIM and RIH in two *unc-86*-null alleles (Table 1).

tph-1::gfp also was not detectable in NSM of *unc-86* mutants. However, NSM continue to accumulate serotonin in *unc-86*-null mutants. Our genetic and pharmacological analyses indicate that the serotonin accumulation in NSM in an *unc-86* mutant is due to import from the ADF neurons (Fig. 3; Table 1): first, NSM serotonin immunoreactivity was significantly reduced in *unc-86* mutants treated with serotonin reuptake block drugs; second, accumulation of NSM serotonin in *unc-86* mutants was much reduced in a genetic background that reduces serotonin levels in ADF. Thus, *unc-86* is a major component in the specification of serotonin in HSN, AIM, RIH and NSM development.

By contrast, *unc-86*-null mutations did not affect serotonin accumulation or *tph-1::gfp* expression in the pair of the ADF neurons that do not express UNC-86. This indicates that different regulatory mechanisms specify serotonergic phenotypes in ADF and UNC-86-expressing neurons. In support of this model we found distinct *cis*-regulatory regions mediating *tph-1* expression in ADF and UNC-86-expressing cells (Fig. 4) and that the *nss-1(yz12)* mutation affects *tph-1::gfp* expression and serotonin accumulation in ADF but has no effect on NSM and HSN (Fig. 3; Table 1). The neuron-specific regulatory mechanism of serotonergic phenotypes has also been identified in *Drosophila*. The LIM-homeobox gene *islet* is expressed in and required for serotonin synthesis in the neurons in the ventral nerve cord but not the serotonergic neurons in the CNS (Thor and Thomas, 1997). It will be interesting to determine whether functionally conserved serotonergic neurons are specified by conserved regulators.

unc-86-null mutations also affect the expression of the *cat-1* vesicular monoamine transporter in NSM, RIH, AIM and HSN (Fig. 2). Reduced *cat-1::gfp* expression was not observed in *tph-1* mutants that are incapable of synthesizing serotonin. Thus, reduced *cat-1* expression is unlikely to be the secondary consequence of reduced serotonin production; rather, *unc-86* coordinately regulates the expression of genes for serotonin synthesis and its packaging into vesicles. In mammals, the ETS domain protein PET-1 is expressed in serotonergic neurons and binds to the promoter of tryptophan hydroxylase and monoamine vesicular transporter genes (Hendricks et al., 1999). The role of PET-1 in vivo is not known. It is possible that one of the *C. elegans* ETS domain proteins (*C. elegans* sequencing Consortium, 1998) also regulates *tph-1* and *cat-1* for example in ADF, or acts as an UNC-86 co-factor to regulate *tph-1* and *cat-1*.

Coordinated transcriptional regulation of neurotransmitter synthesis and packaging may represent a general principle for specification of neurotransmitter phenotypes. In mammals, *Drosophila* and *C elegans*, genes for acetylcholine synthesis and its vesicular transporter are co-regulated by a common promoter (Rand, 1989; Eiden, 1998; Kitamoto et al., 1998). Similarly, the *C elegans* homeodomain transcription factor UNC-30 binds to and is essential for the expression of both the *unc-25*/GABA synthetic enzyme glutamate decarboxylase and *unc-47*/GABA vesicular transporter in the type D motoneurons (Eastman et al., 1999). *unc-86*-null mutations appear to have a stronger effect on the expression of *tph-1* than on *cat-1*. Serotonin can be released by both vesicular and non-vesicular release (for reviews, see Attwell et al., 1993; Bunin and Wightman, 1999). It is conceivable that non-vesicular release is modulated by the rate of serotonin production and vesicular

release is regulated by neuronal stimuli. *unc-86* gene activity may coordinately regulate serotonin synthesis and non-vesicular release, and *unc-86* and other factors act together to regulate vesicular release in response to specific signals. Interestingly, Hobert et al. (Hobert et al., 1999) found that unlike the essential role that *unc-30* plays in the specification of type D GABAergic motoneurons, deletion of LIM-6 strongly affects *unc-25*/glutamate decarboxylase expression but only has a mild effect on *unc-47*/GABA vesicular transporter expression in the head and tail GABAergic neurons. Thus, cell-specific transcriptional regulation may be a mechanism to regulate cell-specific neurotransmission. UNC-86, LIM-6 and UNC-30 are continuously expressed throughout the life of the animal (Finney and Ruvkun, 1990; Hobert et al., 1999; Jin et al., 1994). It remains to be shown that these regulatory mechanisms that specify neurotransmitter phenotypes during the development of the nervous system continuously regulate neurotransmission in mature neurons.

unc-86 is not essential for serotonin reuptake

Unlike the role in the control of serotonin synthesis, *unc-86* appears non-essential for the development of serotonin reuptake machinery. Serotonin released into the synapse is inactivated primarily by reuptake of the transmitter through the serotonin transporter (SERT) present in the plasma membrane of the serotonergic neurons (Amara and Kuhar, 1993). The antidepressents imipramine and fluoxetine block the membrane transporter that reuptakes serotonin and are, thus, thought to enhance effective serotonin signals (Baldessarini, 1996). We found that imipramine and fluoxetine dramatically reduced serotonin accumulation in NSM of *unc-86* mutants (Fig. 3E). These data strongly suggest that *unc-86* mutations disrupt the synthesis of serotonin but do not impair the ability to uptake serotonin.

The argument that imipramine and fluoxetine block NSM uptake of serotonin is further supported by our demonstration that NSM do not accumulate serotonin if *unc-86* mutants also carry a mutation that decreases serotonin biosynthesis in ADF. However, NSM are not connected to ADF (White et al., 1986), thus NSM may uptake serotonin released non-synaptically from ADF. Serotonin release and reuptake are major drug targets to treat depression, eating disorders and obesity, but genetic and molecular basis of the regulatory mechanisms, as well as drug action is poorly understood. Because in *unc-86* mutant background NSM do not accumulate serotonin unless they transport extracellular serotonin released from ADF, isolation of mutations disrupting serotonin accumulation in NSM of *unc-86* mutants will provide a powerful system to define the genetic basis of serotonin release and reuptake.

unc-86 acts late in the serotonergic neural differentiation

Although *unc-86* specifies cell-fates of a number of neural lineages (Chalfie et al., 1981; Finney et al., 1988), *unc-86*-null mutations do not transform the cell fates of the serotonergic neurons. In *unc-86*-null mutants, these neurons are generated, the locations of the cell bodies of NSM, RIH and AIM are normal throughout the life of the animal, overall morphology is largely unaffected, and the position of HSN is also normal in the young larvae (Desai et al., 1988). Furthermore, in the absence of UNC-86, NSM, RIH and AIM still express *gtpch-1* (which encodes a GTP-cyclohydrolase 1, a co-factor of serotonin synthetic enzyme tryptophan hydroxylase), and NSM are able to transport extracellular serotonin. These observations support the notion that early aspects of the neural differentiation of these serotonergic neurons are properly executed in *unc-86*-null mutants, and the function of UNC-86 is confined in late differentiation to regulate specific aspects of their terminal neuronal identity.

We observed a range of abnormal neurite outgrowth from NSM in *unc-86*-null mutants. Extra processes were extended from the cell body, and the main axonal processes were prematurely terminated (Fig. 5B-E). This abnormal neurite outgrowth is unlikely to be the result of decreased serotonin signaling because such defects were not observed in *tph-1* or *cat-1* mutants (Duerr et al., 1999; Sze et al., 2000). Rather, we favor the hypothesis that UNC-86 regulates the expression of signaling components that detect and respond to guidance cues, and structural proteins that mediate neurite extension.

The vertebrate *unc-86* orthologs, *Brn3a* and *Brn3b* selectively regulate the expression of genes involved in neurite outgrowth and pathfindings. It has been shown that in the ND7 cell line, *Brn3a* stimulates the expression of the synaptic vesicle protein SNAP-25 and induces axon outgrowth, but *Brn3b* inhibits SNAP-25 expression (Lakin et al., 1995; Smith et al., 1997b). However, *Brn3b* stimulates the actin-binding protein AbLIM and mice bearing a *Brn3b* deletion mutation exhibit aberrant neurite outgrowth and pathfinding in the retina (Erkman et al., 2000). We have previously observed that activation of UNC-86 by fusion of UNC-86 and the VP16 activation domain causes defects in neurite navigation of the touch receptors (Sze et al., 1997). The pathfinding defects of NSM in *unc-86*-null appear to be different from that of touch receptor neurons in UNC-86/VP16 animals. The NSM posteriorly directed processes tend to be missing or prematurely terminated in *unc-86*-null mutants, whereas the major processes of the touch receptors are abnormally extended or wander out in UNC-86/VP16 animals. It is possible that *unc-86* exerts different function on the expression of genes that mediate neurite navigation in different neurons. Alternatively, proper levels of guidance molecules and structural proteins may be essential for correct pattern of neurite navigation, thus either increased expression levels in UNC-86/VP16 animals or decreased expression levels in *unc-86*-null mutants would cause incorrect interpretation of guidance cues and result in neurite outgrowth defects.

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