Caenorhabditis elegans TRPV ion channel regulates 5HT biosynthesis in chemosensory neurons

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Summary

Serotonin (5HT) is a pivotal signaling molecule that modulates behavioral and endocrine responses to diverse chemical and physical stimuli. We report cell-specific regulation of 5HT biosynthesis by transient receptor potential V (TRPV) ion channels in *C. elegans*. Mutations in the TRPV genes *osm-9* or *ocr-2* dramatically downregulate the expression of the gene encoding the 5HT synthesis enzyme tryptophan hydroxylase (*tph-1*) in the serotonergic chemosensory neurons ADF, but neither the mutation nor the double mutation of both channel genes affects other types of serotonergic neurons. The TRPV genes are expressed in the ADF neurons but not in other serotonergic neurons, and act cell-autonomously to regulate a neuron-specific transcription program. Whereas in olfactory neurons OSM-9 and OCR-2 function is dependent on ODR-3 Gα, the activity of ODR-3 or two other Gα proteins expressed in the ADF neurons is not required for upregulating *tph-1* expression, thus the TRPV ion channels in different neurons may be regulated by different mechanisms. A gain-of-function mutation in CaMKII UNC-43 partially suppresses the downregulation of *tph-1* in the TRPV mutants, thus CaMKII may be an effector of the TRPV signaling. Mutations in the TRPV genes cause worms developmentally arrest at the Dauer stage. This developmental defect is due in part to reduced 5HT inputs into *daf-2*/insulin neuroendocrine signaling.

Key words: *C. elegans*, Transcription, Serotonin biosynthesis, TRPV ion channels, Metabolic control

Introduction

The monoamine 5HT acts as a neurotransmitter and a hormone to induce behavioral and endocrine responses to changes in environmental and physiologic states. A deficit in 5HT is implicated in a broad spectrum of disorders such as depression, eating disorders and type II diabetes (reviewed by Lucki, 1998; Leibowitz and Alexander, 1998; Davidson et al., 2000). In both vertebrates and invertebrates, 5HT is synthesized in a set of neurons with diverse synaptic properties and connectivities. Activation of serotonergic neurons by sensory stimuli was first clearly demonstrated in 1976 through studies in *Aplysia*, where sensory stimuli increase 5HT signals to induce synaptic facilitation and behavioral sensitization (Brunelli et al., 1976). Long-standing questions are: how do serotonergic neurons integrate sensory signals into 5HT neurotransmission, and are different serotonergic neurons regulated by cell-specific molecular mechanisms?

TRP-related proteins are a superfamily of cation channels that share structural homology to the *Drosophila* transient receptor potential (TRP) protein (reviewed by Clapham et al., 2001; Montell et al., 2002). TRP channels act as molecular integrators of a wide range of chemical and physical stimuli to regulate behavior and physiological function in both vertebrates and invertebrates (reviewed by Scott and Zuker, 1998; Minke and Cook, 2002). Five genes in the *C. elegans* genome, *ocr-1*, *ocr-2*, *ocr-3*, *ocr-4* and *osm-9*, encode TRPV subfamily proteins characterized by cytoplasmic N-terminal multiple ankyrin repeats, six transmembrane segments and a non-conserved cytoplasmic C terminus (Harteneck et al., 2000; Tobin et al., 2002). These TRPV genes are expressed in the sensory endings of chemosensory neurons and have been shown to regulate sensory functions and social behavior (Colbert et al., 1997; Tobin et al., 2002; de Bono et al., 2002). In mammals, TRPV channels transmit nociceptive stimuli such as pain (Tominaga et al., 1998) and noxious heat (Caterina et al., 1997; Caterina et al., 1999). In addition, mammalian TRPV ion channels also mediate the response to growth factors (Kanzaki et al., 1999). Agonists to TRPV channels induce hypothermia (Meller et al., 1992; Szallasi and Blumberg, 1996) and modulate oxygen consumption (Colquhoun et al., 1995). Mammalian TRPV proteins are expressed in the sensory neurons, as well as in the CNS (Tominaga et al., 1998; Hayes et al., 2000; Mezey et al., 2000; Delany et al., 2001). It has been postulated that TRPV ion channels regulate the release of neural mediators to modulate endocrine activity (Szallasi and Blumberg, 1996), but in vivo evidence has not yet been reported.

We describe the effect of mutations in two TRPV ion channel genes, *osm-9* and *ocr-2*, on biosynthesis of 5HT in *C. elegans*. Previously, we demonstrated that the *tph-1* gene, which encodes the key 5HT biosynthesis enzyme tryptophan hydroxylase, is essential for 5HT biosynthesis and that *tph-1* expression is regulated by cell-specific mechanisms (Sze et al., 2000; Sze et al., 2002). In this study we show that a signaling pathway involving the *osm-9* and *ocr-2* TRPV channel proteins, CaMKII specifically regulates *tph-1* expression in the

serotonergic chemosensory neurons ADF. Results from this study reveal a remarkably neuron-specific mechanism regulating 5HT biosynthesis and provide genetic insights into how a neuron transduces the events at the cell-surface to 5HT signaling.

Materials and methods

Worm strains

The strains used in this study were wild-type *C. elegans* Bristol strain (N2) and mutants *osm-9(yz6)*, *osm-9(n2743)*, *osm-9(ky10)*, *osm-9(n1516)*, *ocr-2(yz5)*, *ocr-2(ak47)*, *odr-10(ky225)*, *osm-11(n1604)*, *tph-1(mg280)*, *daf-7(e1372)*, *daf-16(mgDf50)*, *nss-1(yz12)*, *odr-3(n2150); gpa-3(pk35); gpa-13(pk330); unc-43(n498)gf* and *unc-43(e408)*. The worms were fed with *E. coli* OP50 as the food.

Isolation and characterization of *osm-9(yz6)* and *ocr-2(yz5)* mutants

The *yz6* and *yz5* mutations were isolated based on reduction/absence of GFP in the ADF neurons after ethylmethane sulfonate mutagenesis of wild-type animals carrying an integrated *tph-1::gfp* transgene. The mutagenesis and mutant screens have been described previously (Sze et al., 2002). We screened about 6500 haploid genomes and isolated 24 mutants. None of the mutations completely eliminates *tph-1::gfp* expression in the ADF neurons, but *yz12* (Sze et al., 2002), *yz5*, *yz6* and other three mutants showed stronger effects. Genetic mapping and complementation analysis indicated all these six strong mutants as single alleles, thus the screen is probably unsaturated. We mapped *yz6* between the polymorphisms in the clones C09G12 and M02B7, and *yz5* between C49H3 and C01F6. A PCR fragment of the cosmid M57 containing 2.8 kb of the upstream sequence, exons/introns and 1.3 kb downstream sequence of *osm-9* restores *tph-1::gfp* expression in ADF of *yz6* animals. A PCR fragment of the cosmid T09A12 containing 2.5 kb upstream sequence, exons/introns and 1.8 kb downstream sequence of *ocr-2* restores *tph-1::gfp* expression in *yz5* mutants. The molecular lesion of the mutations was determined by PCR amplification of the exons and exon/intron boundaries from the mutant strains and sequencing the PCR fragments.

Expression of *osm-9* from heterologous promoters

The *osm-9* and *ocr-2* genes are co-expressed in six pairs of neurons: ADF, AWA, ADL, ASH, PHA and PHB (Colbert et al., 1997; Tobin et al., 2002). Chimeric constructs were generated by expressing wild-type *osm-9*- or *ocr-2*-coding regions under the control of heterologous promoters that are expressed in a subset of these six pairs of the neurons. The expression pattern of the heterologous promoters overlaps with *osm-9* and *ocr-2* in the following neurons: *odr-7*, AWA (Sengupta et al., 1994); *osm-10*, ASH, PHA and PHB (Hart et al., 1999); *tax-2*, PHA and PHB (Coburn and Bargmann, 1996); *cat-1*, ADF (Sze et al., 2002); *lin-11*, ADF, ADL (Hobert et al., 1998); *tph-1(BC)*, ADF (this work). In each case, we first constructed a fusion of the promoter region to the GFP and *unc-54* 3′-uncoding sequences in the plasmid pPD97.75 (A. Fire) to confirm the expression pattern, then, we replaced the GFP sequence with a genomic sequence encompassing the entire intron and exon regions of *osm-9* or *ocr-2*. Individual constructs were introduced into *yz6* or *yz5* mutants carrying the integrated *tph-1::gfp* reporter. The *tph-1*(BC) construct was generated by inserting the sequence from –132 to –377 upstream of the *tph-1* translational start [the BC region as described previously (Sze et al., 2002)] to a minimal promoter of the *pes-10* gene in the GFP vector pPD122.53 (A. Fire). The plasmid pRF4 containing the dominant Rol-6 gene was co-injected as a transgenic marker for Promoter::gfp constructs, and a plasmid containing *elt-2::gfp* (a gift from J. McGhee) as the marker for Promoter::*osm-9* and Promoter::*ocr-2* constructs.

GFP expression and Immunoanalysis

The expression pattern of these GFP transgenes in wild-type animals has been published: the *tph-1::gfp*, *lin-11::gfp* and *cat-1::gfp* transgenes were integrated into the chromosomes, and *gtpch-1::gfp* was carried as extra chromosomal arrays (Hobert et al., 1998; Sze et al., 2000; Sze et al., 2002). The individual transgenes were crossed into mutants. Thus, the expression of the same transgene in wild-type and mutant animals was compared.

To quantify GFP intensity of *tph-1::gfp* in the ADF neurons, the images were captured with a Zeiss AxioCam digital camera at a fixed exposure time, and the fluorescence within a 25×25 pixel area of the cell body was scored, hence the same dimension of the ADF neurons in different genetic background was compared.

The staining of anti-5HT antibody was performed using the McIntire-Horvitz whole-mount procedure with modifications as described (McIntire et al., 1992; Sze et al., 2000).

Behavioral assays

Feeding and egg-laying assays were conducted with young adult animals. Well-fed larval stage 4 animals (L4) were picked onto fresh plates seeded with bacteria as the food, and allowed to develop ~20 hours at 20°C. Feeding behavior was assayed by measuring the rate of pharyngeal pumping, which was scored by counting pharynx terminal bulb contractions (Duerr et al., 1999; Sze et al., 2000). Egg-laying behavior was scored by counting the number of fertilized eggs accumulated inside of the uterus of adults, using DIC optics (Sze et al., 2000).

For Dauer assays, 10 young adult animals of a strain were transferred onto a fresh plate and allowed to lay eggs for overnight. The parents were then removed, progeny were allowed to develop at 15°C, and the number of Dauers and L4/adults was scored 5-6 days later. Notice a higher Dauer frequency of *tph-1;daf-7* double mutant animals than we previously reported (Sze et al., 2000). This difference is due to different time points at which we scored Dauers. *tph-1;daf-7* mutants grow slower, some of the animals were still at pre-Dauer stages at the earlier time point.

Results

yz5 and yz6 mutations specifically affect the production of 5HT in the pair of the chemosensory neurons ADF

Of the 302 neurons present in an adult *C. elegans* hermaphrodite, nine neurons from five distinct classes are detected by antibodies raised against 5HT (Horvitz et al., 1982) (Fig. 1A); four classes exist as left-right symmetric pairs: the ADF chemosensory neurons, the NSM pharyngeal secretory neurons, the HSN motor neurons, and the AIM interneurons and RIH is a single interneuron. These neurons are generated from different lineages during embryogenesis (Sulston et al., 1983). 5HT immunoreactivity can be detected in the ADF, NSM, AIM and RIH neurons shortly after hatching, and in the HSN neurons only in adults. The ability to monitor identified serotonergic neurons permits the isolation and analysis of mutant genes affecting serotonergic phenotype in specific neurons.

Biosynthesis of 5HT in *C. elegans* requires the *tph-1* gene, which encodes the enzyme tryptophan hydroxylase catalyzing the rate-limiting first step of 5HT biosynthesis. *tph-1* is expressed in serotonergic neurons, and *tph-1* knockout animals have no detectable 5HT (Sze et al., 2000). Our previous study indicated that *tph-1* expression in different serotonergic neurons is regulated by distinct transcription programs (Sze et al., 2002). To define genes underlying this neuron-specific

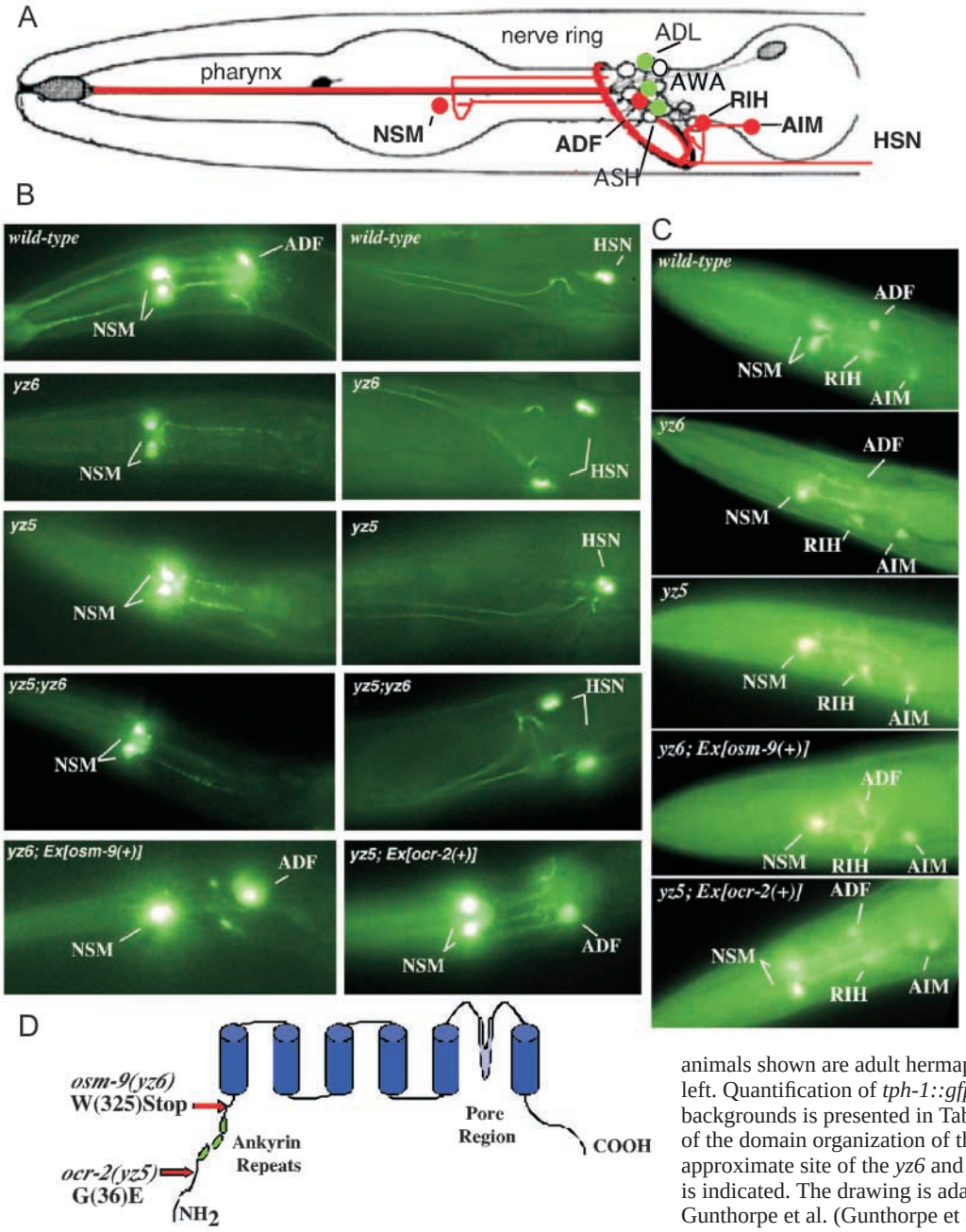

Fig. 1. Cell-specific effects of *yz5* and *yz6* mutations on 5HT biosynthesis. (A) The position of serotonergic neurons in the head, and the axon from HSN (shown in red). Also shown are the non-serotonergic amphid chemosensory neurons that express both *osm-9* and *ocr-2* (in green) (Tobin et al., 2002). The drawing is adapted, with permission, from Starich et al. (Starich et al., 1995). (B) GFP expression of an integrated *tph-1::gfp* fusion gene in wild-type and mutant animals. In wild type, GFP is strongly expressed in the ADF, NSM and HSN neurons. *yz5* and *yz6* mutations specifically downregulate the GFP expression in the ADF chemosensory neurons. Note that neither the mutation alone, nor *yz5;yz6* double mutation affects GFP levels in NSM and HSN. *tph-1::gfp* expression in ADF is restored in the mutant animals carrying a wild-type *osm-9* or *ocr-2* transgene, respectively. (C) Anti-5HT antibody staining of wild-type and mutant animals. *yz5* and the *yz6* mutant animals show reduction or absence of 5HT immunoreactivity in the ADF neurons. ADF 5HT immunoreactivity is recovered in the mutants carrying a wild-type *osm-9* or *ocr-2* transgene. All the animals shown are adult hermaphrodites. Anterior is towards the left. Quantification of *tph-1::gfp* expression in various genetic backgrounds is presented in Table 1. (D) A schematic representation of the domain organization of the TRPV subfamily. The approximate site of the *yz6* and *yz5* mutation in the channel structure is indicated. The drawing is adapted, with permission, from Gunthorpe et al. (Gunthorpe et al., 2002).

regulation of 5HT synthesis, we conducted a genetic screen for neuron-specific serotonin defective (*nss*) mutants, using a green fluorescent protein (GFP) fusion to *tph-1* (*tph-1::gfp*) as a reporter.

yz5 and *yz6* are two of the *nss* mutations that specifically downregulate *tph-1* expression in the ADF neurons. In wild-type animals, *tph-1::gfp* is highly expressed in the ADF, NSM and HSN neurons, but the GFP level in the ADF neurons is dramatically reduced or undetectable in *yz5* and *yz6* mutants (Fig. 1B, Table 1). Consistent with the essential role of *tph-1* in 5HT biosynthesis, staining of the mutant animals with anti-5HT antibody shows reduced/absence of 5HT immunoreactivity in the ADF neurons (Fig. 1C). However, neither the mutation nor *yz5;yz6* double mutation has a detectable effect on *tph-1::gfp* expression or 5HT immunoreactivity in other serotonergic neurons (Table 1; Fig. 1B,C). The ADF neurons are the only serotonergic sensory neurons in hermaphroditic *C. elegans*, the data suggest that *yz5* and *yz6* specifically regulate the serotonergic phenotype of sensory neurons.

yz5 and *yz6* are mutations of the *ocr-2* and *osm-9* TRPV channel genes, respectively

Genetic mapping and transgene rescue of *yz5* and *yz6* mutations revealed two TRPV channel proteins regulating *tph-1* expression in serotonergic chemosensory neurons. The TRPV subfamily is characterized by the cytoplasmic N-terminal multiple ankyrin repeats, six transmembrane segments and a cytoplasmic C terminus (Harteneck et al., 2000) (Fig. 1D). Our genetic mapping and sequencing of the mutant

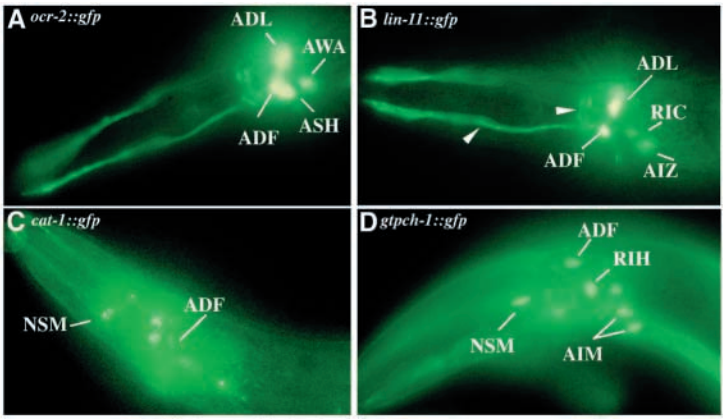

Fig. 2. Mutations in the TRPV channel proteins do not cause general cell fate transformation of the ADF neurons. (A) Expression of a *ocr-2::gfp* reporter in a wild-type animal. Both *osm-9* and *ocr-2* are expressed in ADF (Tobin et al., 2002), but not in other serotonergic neurons. (B-D) Expression of ADF markers in *osm-9(yz6)* and *ocr-2(yz5)* mutants. The GFP reporters were examined in wild-type, *yz6* and *yz5* mutant animals. The expression patterns in wild-type animals have been published: *lin-11:gfp* (Hobert et al., 1998), and *cat-1::gfp* and *gtpch-1::gfp* (Sze et al., 2002). Representative examples of the reporter expression patterns in mutant animals are shown. (B) The expression of *lin-11::gfp* overlaps with *osm-9* and *ocr-2* in the ADF and ADL chemosensory neurons. Shown is a *yz6* mutant animal strongly expressing *lin-11::gfp* in ADF and ADL. The position of the cell body and the morphology of the axon and dendrite are normal (indicated by arrowheads). (C) A GFP reporter of the CAT-1/vesicular monoamine transporter is localized to the synapses of the serotonergic neurons and dopaminergic neurons (Sze et al., 2002). Unlike the dramatic reduction of *tph-1::gfp* expression in the ADF neurons (Fig. 1B, Table 1), *cat-1::gfp* can be clearly observed in the ADF neurons of *yz5* and *yz6* mutants. We have repeatedly observed that *cat-1::gfp* expression in the ADF neurons is relatively weaker in *yz5* and *yz6* mutants than in wild-type animals, but the differences were not statistically significant. The GFP is distributed as punctate pattern, presumably the fusion CAT-1 is associated with synaptic components. (D) A GFP reporter of a probable GTP-cyclohydrolase I gene *gtpch-1* is strongly expressed in serotonergic and dopaminergic neurons (Sze et al., 2002). Shown is a *yz6* animal expressing *gtpch-1::gfp* in ADF and in other serotonergic neurons.

genomic DNA show that *yz6* is a nonsense mutation that results in a stop codon before the transmembrane domain in the *osm-9* TRPV gene, and *yz5* is a missense mutation adjacent to the conserved ankyrin motifs in *ocr-2* (Fig. 1D). To confirm it is the mutation in the TRPV genes that downregulates *tph-1* expression in the ADF neurons, we examined *tph-1::gfp* expression and 5HT immunoreactivity in the *ocr-2* deletion mutant *ak47* and three *osm-9* alleles (*ky10*, *n2743*, *n1516*). In every of the mutant strains, *tph-1::gfp* expression in ADF is downregulated and 5HT immunoreactivity in ADF is reduced (Table 1; data not shown). Furthermore, *yz6* and *yz5* mutant animals carrying a transgene containing the wild-type *osm-9* or *ocr-2* gene, respectively, restore *tph-1::gfp* expression and 5HT immunoreactivity (Fig. 1B,C; Table 1). We conclude that *yz6* is an allele of the *osm-9* gene, and *yz5* is an allele of *ocr-2*.

Table 1. *osm-9* and *ocr-2* mutations downregulate *tph-1::gfp* expression in the ADF chemosensory neurons

	% of GFP in ADF					
Strains	Strong	Weak	Very weak/ none	NSM	HSN	*n*
Wild type	98	0	2	100	100	122
yz6	0	16	84	100	100	183
osm-9(ky10)	0	27	73	100	100	118
osm-9(n2743)	0	14	86	100	100	138
osm-9(n1516)	0	9	91	100	100	142
yz5	0	22	78	100	100	235
ocr-2(ak47)	0	31	69	100	100	97
yz6; yz5	0	10	90	100	100	205
yz6; Ex[osm-9(+)]	39	47	14	100	100	301
yz5; Ex[ocr-2(+)]	42	50	8	100	100	123
odr-10(ky225)	99	1	0	100	100	104
osm-11(n1604)	98	2	0	100	100	124

All the strains carry the same integrated *tph-1::gfp* transgene.

Strong, equivalent to GFP in wild-type animals shown in Fig. 1B; weak, GFP still detectable in the cell body; very weak/none, GFP almost visually undetectable.

n is the number of animals examined.

Mixed staged animals were observed; GFP in HSN was scored only in adults.

Percentage of animals in each category is shown.

TRPV ion channels act cell autonomously to control 5HT biosynthesis

Both *osm-9* and *ocr-2* are expressed in the ADF neurons (Colbert et al., 1997; Tobin et al., 2002), but not in other serotonergic neurons (Fig. 2A). Beside ADF, *osm-9* and *ocr-2* also are co-expressed in five pairs of non-serotonergic chemosensory neurons: the AWA, ADL, ASH neurons in the head, and the PHA and PHB neurons in the tail (Tobin et al., 2002). These head neurons, as well as ADF, are component neurons of the amphid sensory organ, and each class senses distinct signals (Bargmann and Horvitz, 1991a). The cell bodies of these head neurons are clustered together, and their processes run parallel and are interconnected directly or indirectly en passant (White et al., 1986) (Fig. 1A). The TRPV channels could act in the ADF neurons to control *tph-1* expression, or they could function in the other chemosensory neurons that regulate ADF neural activity. The AWA neurons detect the attractive odorant diacetyl, and the ASH and ADL neurons sense aversive signals; *ocr-2(ak47)* and *osm-9* mutant animals are defective in sensing these sensory signals (Colbert et al., 1997; Tobin et al., 2002). We tested whether disruption of these sensory signaling is a cause of reducing *tph-1::gfp* expression. No GFP reduction was observed in animals with defective diacetyl receptor (*odr-10*) (Sengupta et al., 1996) or with defective ASH, ADL (*osm-11*) function (Table 1). Thus, *osm-9/ocr-2* function in these sensory signaling is not required to activate *tph-1* expression in the ADF neurons.

To identify cells in which the TRPV proteins function to upregulate *tph-1* expression, we constructed a series of chimeric genes with the *osm-9*- or *ocr-2*-coding regions under the control of heterologous promoters and generated transgenic animals expressing wild-type *osm-9* or *ocr-2* proteins in a subset of these six neuronal types. When *osm-9(yz6)* and *ocr-2(yz5)* mutant animals carry a transgene expressed in the ADF

Table 2. *osm-9* acts in the ADF chemosensory neurons to upregulate the *tph-1* expression

Transgene carried in *osm-9(vz6)* mutants	Transgene expression in ADF	% of *tph-1::gfp* in ADF			
		Strong	Weak	Very weak/ none	*n*
None	–	0	16	84	183
Ex [*Podr-7::osm-9*]	No	0	4	96	31
Ex [*Posm-10::osm-9*]	No	0	14	86	111
Ex [*Ptax-2::osm-9*]	No	0	15	85	100
Ex [*Pcat-1::osm-9*]	Yes	70	28	2	166
Ex [*Plin-11::osm-9*]	Yes	64	35	1	94
Ex [*Ptph-1(BC)::osm-9*]	Weakly	36	23	41	187

The same genomic *osm-9* sequence was fused to individual heterologous promoter fragments. The expression patterns were confirmed by construction of Promoter::gfp fusion. Although Ptph-1(BC) is expressed strongly in ADF of wild-type animals, the ADF expression is much weaker in *osm-9* mutant background (Fig. 3A).

All the transgenes were carried as extrachromosomal arrays, three to five transgenic lines were examined for each construct. For each line, the animals carrying the marker for the fusion construct and their non-transgenic sibling were examined to ensure *tph-1::gfp* expression in the non-transgenic animals was unaffected. The animals carrying the marker for the fusion constructs were scored.

The data are the summary of at least three independent trials from mutiple generations of the animals. *n* is the number of animals carrying the fusion transgene examined. The definition of the GFP strength is described in Table 1. Percentage of animals in each category is shown.

neurons, *tph-1::gfp* expression in the ADF neurons is restored, whereas the mutant animals carrying the transgene not expressed in the ADF neurons exhibit reduced *tph-1::gfp* expression similar to their non-transgenic siblings (Table 2). These observations collectively argue that the *osm-9* and *ocr-2* genes act cell-autonomously in the ADF neurons to control the *tph-1* expression.

TRPV ion channels regulate specific aspects of serotonergic phenotype

Mutations in OSM-9 and OCR-2 channels may disrupt the regulatory pathway of *tph-1* transcription, or they may induce ADF neural degeneration due to ion imbalances. To address these possibilities, we assessed if the *yz6* and *yz5* mutations result in a general morphological transformation of the ADF neurons. The LIM-homeodomain transcription factor *lin-11* is co-expressed with *osm-9* and *ocr-2* in the ADF and ADL chemosensory neurons (Freyd et al., 1990; Hobert et al., 1998). We crossed an integrated *lin-11::gfp* transgene into *osm-9(yz6)* and *ocr-2(yz5)* mutants; no reduction of *lin-11::gfp* expression levels can be detected in the mutant backgrounds (Fig. 2B). Judged by fluorescence microscopy, the morphology of the cell body and the processes of the ADF neurons are indistinguishable in the mutant and wild-type animals at any developmental stage. Thus, these mutations do not cause the ADF neurons to die prematurely. Rather, these results point to a signaling pathway downstream of the OSM-9 and OCR-2 TRPV channels regulating the transcription of *tph-1* in the ADF neurons.

Does this TRPV channel signaling specifically regulate *tph-1* expression, or does it regulate the expression of all genes involved in 5HT synthesis and neurotransmission? We have explored this question by examining GFP reporters of marker genes. In each case, the GFP reporter construct was first introduced into wild-type animals, and the resulting transgene was then crossed into mutants. Thus, the expression of the same transgene in different genetic backgrounds was compared. CAT-1/vesicular monoamine transporter is required for 5HT neurotransmission (Duerr et al., 1999; Nurish 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 localized to the synapses of the serotonergic neurons (Sze et al., 2002). The gene F32G8.6 encodes a probable GTP-cyclohydrolase I (*gtpch-1*), a co-factor of tryptophan hydroxylase for 5HT biosynthesis, and a *gtpch-1::gfp* fusion gene also is expressed in the serotonergic neurons (Sze et al., 2002). Unlike the dramatic reduction of *tph-1::gfp* in the ADF neurons, there is no significant reduction of *cat-1::gfp* or *gtpch-1::gfp* in *yz6* and *yz5* mutant backgrounds (Fig. 2C,D). These observations are consistent with our previous results that the expression of *tph-1*, *cat-1* and *gtpch-1* is differentially regulated in serotonergic neurons (Sze et al., 2002). These data demonstrate a great specificity of the TRPV channel signaling within the ADF neurons and suggest the transcriptional regulation of the *tph-1* gene as a major target.

The TRPV channel signaling modulates a neuron-specific transcription program

Analysis of the *tph-1* promoter has revealed a discrete cis-regulatory region essential for *tph-1* expression in the ADF neurons (Sze et al., 2002). We investigated whether the TRPV ion channel signaling acts through this neuron-specific transcriptional regulatory mechanism. A GFP reporter under the control of the sequence –132 bp to –377 bp of *tph-1* and a minimal promoter from the *pes-10* gene is specifically expressed in the ADF neurons of wild-type animals, but the ADF GFP intensity is significantly reduced in *yz6* mutant background (Fig. 3A). Thus, the 246 bp cis-regulatory region is sufficient to activate *tph-1* expression in the ADF neurons, and signaling from the OSM-9 and OCR-2 ion channels regulates the activity of this neuron-specific transcriptional regulatory program.

Unlike mutations in the POU-transcription factor UNC-86 that completely abolish *tph-1::gfp* expression in the NSM and HSN neurons (Sze et al., 2002), none of the mutations in *osm-9* or *ocr-2*, nor the mutation of both eliminates *tph-1::gfp* expression in the ADF neurons (Table 1; Fig. 3B), indicating that the TRPV activity modulates *tph-1* expression levels but is not essential for the transcription. In *yz6* background, the GFP reporter under the control of the 246-bp *tph-1* cis-regulatory sequence fused to a *pes-10* minimal promoter is expressed in the ADF neurons at slightly higher levels than the *tph-1::gfp* reporter under the control of the 3.1 kb of the *tph-1* promoter (Fig. 1B, Fig. 3A). This could reflect the difference in the basal expression level of the *tph-1* and *pes-10* promoter in the reporter constructs. Alternatively, it could be an indication of additional cis-regulatory elements mediating inhibition of *tph-1* expression but the elements are not present in the 246 bp *tph-1* sequence.

unc-43 CaMKII acts downstream or in parallel with the TRPV ion channels to modulate 5HT biosynthesis

The *odr-3* Gα protein is essential for *osm-9* and *ocr-2* function in olfactory, osmosensory and mechanosensory behaviors

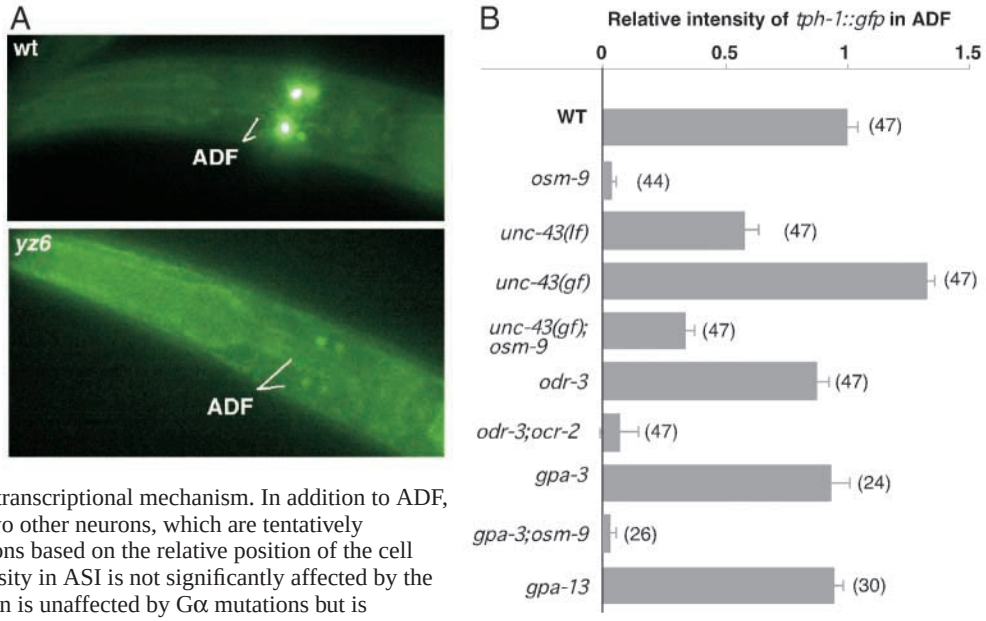

Fig. 3. TRPV channel-dependent regulation of *tph-1* expression is mediated by a specific cis-regulatory region of *tph-1* and is modulated by *unc-43* CaMKII. (A) Expression of a GFP reporter under the control of the sequence –132 to –377 of *tph-1* and a *pes-10* minimal promoter in wild-type and *osm-9* mutant animals. In wild-type animals, GFP is strongly expressed in the ADF neurons, but not in any other serotonergic neuron, indicating that this *tph-1* cis-regulatory region specifically mediates *tph-1* expression in the ADF neurons. The ADF expression of this GFP reporter is significantly reduced in *yz6* mutant animals; hence, the TRPV signaling stimulates this neuron-specific transcriptional mechanism. In addition to ADF, this GFP reporter is often expressed in two other neurons, which are tentatively identified as the ASI chemosensory neurons based on the relative position of the cell body (White et al., 1986). The GFP intensity in ASI is not significantly affected by the TRPV mutation. (B) *tph-1::gfp* expression is unaffected by Gα mutations but is modulated by *unc-43* CaMKII activity. All the strains bear the same GFP reporter. The average of ADF GFP intensity in wild-type animals is defined as 1, and the average GFP intensity in other strains is normalized against the wild-type average. The data are the summary of four independent trials, and the total number of animals scored for each strain is indicated next to the bar. Error bars indicate the mean of the standard error (s.e.m.).

mediated by the AWA and ASH neurons (Roayaie et al., 1998; Colbert et al., 1997; Tobin et al., 2002). It has been proposed that ODR-3 activity modulates the outputs of the TRPV channel signaling (Roayaie et al., 1998). *odr-3* also is expressed in the ADF neurons; however, the *odr-3(n2150)* deletion mutation does not cause a significant reduction of *tph-1::gfp* expression (Fig. 3B). Thus, OSM-9 and OCR-2 can activate *tph-1* expression in the absence of *odr-3* activity. Beside *odr-3*, the ADF neurons express two other Gα proteins, *gpa-3* and *gpa-13* (Jansen et al., 1999). *tph-1::gfp* expression is unaffected in *gpa-3* or *gpa-13* deletion mutants (Fig. 3B). However, animals bearing a double mutation of TRPV and Gα still exhibit reduced *tph-1::gfp* expression in the ADF neurons similar to the TRPV mutants. These data indicate that unlike the role of ODR-3 in the sensory behaviors, the Gα proteins do not play an essential role in OSM-9/OCR-2-dependent regulation of *tph-1* expression. Because the sensory behaviors and 5HT production are mediated by different neurons, our results suggest that OSM-9 and OCR-2 TRPV channels in different neurons may be regulated by different mechanisms.

CaMKII is a critical mediator of Ca^{2+} signaling. The *unc-43* gene encodes the only *C. elegans* CaMKII (Reiner et al., 1999; Rongo and Kaplan, 1999). An *unc-43* loss-of-function mutation causes a twofold reduction of *tph-1::gfp* expression in the ADF neurons (Fig. 3B), but has no effect on other serotonergic neurons (not shown). Conversely, the *unc-43(n498)* gain-of-function mutation partially blocks the downregulation of *tph-1* expression in *yz6* (Fig. 3b) and *yz5* mutants (data not shown). One simple model to explain these data would be that activation of OSM-9 and OCR-2 channels increases ADF intracellular Ca^{2+} which stimulates UNC-43 to induce *tph-1* transcription, whereas the *unc43(n498)* mutation, which causes constitutive Ca^{2+}-independent activity (Reiner et al., 1999), bypasses the need of the channel function. However, these results do not exclude the possibility that UNC-43 acts less directly in the TRPV channel signaling pathway. These results suggest that CaMKII acts downstream of or in parallel with the OSM-9 and OCR-2 TRPV channels to control 5HT production in the ADF chemosensory neurons.

Dauer phenotype of *osm-9* and *ocr-2* mutants

We find that both *osm-9* and *ocr-2* mutants show developmental defects reminiscent of *tph-1* deletion mutants (Fig. 4A). The DAF-2/insulin receptor and DAF-7/TGFβ signaling act in parallel to control whether an animal enters the reproductive lifecycle or developmentally arrests at the metabolically inactive Dauer larval stage. Disruption of either pathway causes conditional abnormal arrest at the Dauer stage, but disruption of both the pathways causes constitutive Dauer arrest (Ogg et al., 1997) (reviewed by Riddle, 1997). Similar to the *tph-1* deletion mutation, the *osm-9(yz6)*; *osm-9(ky10)* and *ocr-2(yz5)* mutations enhance the Dauer phenotype of *daf-7(e1372)* mutants (Fig. 4A). At the 15°C growth temperature, about 10% of *daf-7* mutant animals arrest as Dauers, but more than 70% form Dauers when *daf-7* mutants carry a mutation in *osm-9* or *ocr-2*. Ninety-eight percent of *daf-7;tph-1* double mutant animals form Dauers, whereas 10-15% *tph-1* mutants form Dauers (Sze et al., 2000). None of the TRPV mutants on their own formed Dauers when assayed under the same condition, although they grow slower than wild-type animals. Because *e1372* is a *daf-7*-null mutation, this enhanced Dauer phenotype of the double mutants implies that the TRPV-mutations affect a pathway parallel to *daf-7*.

Enhancement and suppression genetics and other molecular experiments implicate that the TRPV mutations affect 5HT inputs to the insulin pathway. The DAF-16/forkhead transcription factor is a negative target of DAF-2/insulin signaling, and the *daf-16(mgDf50)* mutation bypasses the need of DAF-2 (Ogg et al., 1997). The Dauer phenotype of *daf-7;*

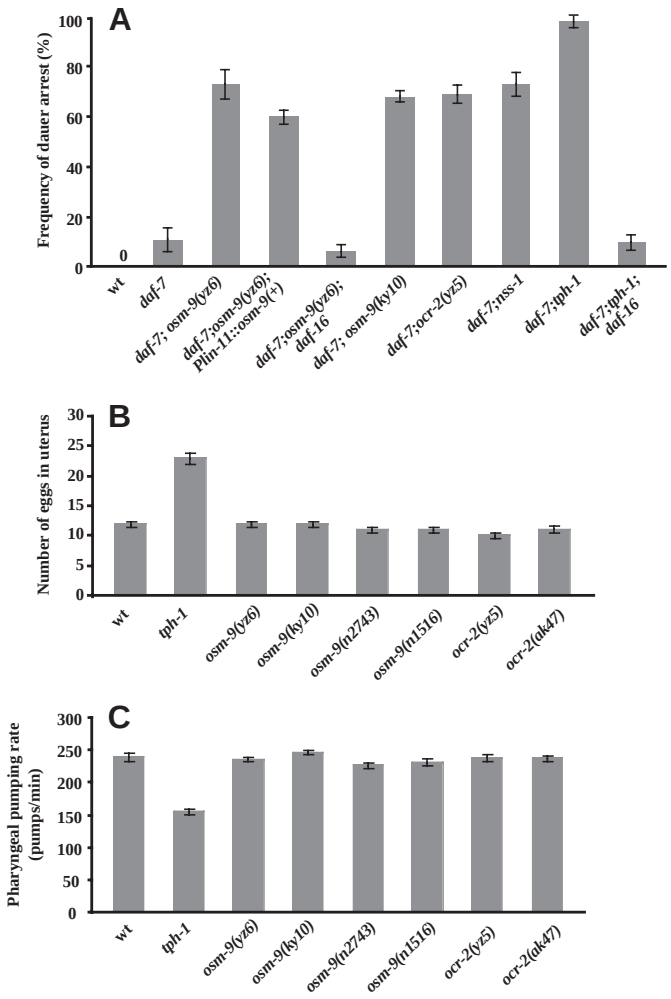

Fig. 4. Effects of the TRPV genes on 5HT-modulated behaviors. (A) Dauer metabolic arrest. Similar to the *tph-1* deletion mutation, the TRPV mutations and another ADF-5HT deficit mutation, *nss-1(yz12)*, enhance Dauer arrest of the *daf-7(e1372)* mutation at the 15°C growth temperature. This enhanced Dauer phenotype is suppressed by a deletion mutation of the *daf-16* gene, which is a negative target of the DAF-2/insulin signaling pathway. We noticed that *daf-7;osm-9;daf-16* animals grow slower and sometimes form Dauer-like larvae but then go on to develop to adults, suggesting that the *daf-2* pathway may not be the only signaling affected in the mutant. The *Plin-11::osm-9(+)* transgene was carried as an extrachromosomal array, only the animals carrying the transgenic marker were scored. The *osm-9(yz6)* (n=956), or *ocr-2(yz5)* (n=346) mutants alone do not form Dauers under the assay condition. (B) Egg-laying behavior. Unlike *tph-1* mutant animals, the TRPV mutant adults do not accumulate a large amount of fertilized eggs in the uterus. (C) Feeding behavior. *tph-1* mutant animals have no detectable 5HT in any serotonergic neuron and exhibit slower pharyngeal pumping rates, whereas animals bearing a mutation in the TRPV genes pump at rates similar to wild-type animals. The feeding and egg-laying behavior represents the summary of two independent trials, ten animals/strain/trial. The Dauer assay is the summary of three independent trials, each in duplicate. Error bars indicate the s.e.m.

tph-1 and *daf-7;osm-9* can be suppressed by the *daf-16(mgDf50)* mutation (Fig. 4A), indicating a reduction of DAF-2/insulin signaling in the double mutants that promotes the Dauer formation. Dauer arrest is also enhanced in *daf-7(e1372)* mutants carrying a mutation in the *nss-1* gene, which also specifically affects *tph-1* expression in ADF (Sze et al., 2002). This raises the possibility that it is the reduction of ADF 5HT signals that downregulates the DAF-2/insulin pathway. However, expression of the wild-type *osm-9*-coding sequence under a *lin-11* promoter only mildly suppresses the Dauer phenotype of *osm-9(yz6);daf-7(e1372)* mutants (Fig. 4A), indicating that other *osm-9*-expressing cells also contribute to the normal development.

osm-9 and *ocr-2* mutant animals do not display every deficit observed in mutants with all the serotonergic neurons defective. 5HT regulates several *C. elegans* behaviors. For example, applying exogenous 5HT to *C. elegans* stimulates pharyngeal pumping and egg-laying (Avery and Horvitz, 1990; Weinshenker et al., 1995), whereas 5HT-deficient mutants *tph-1* and *cat-1* exhibit a slower pumping rate and accumulate a large number of fertilized eggs in the uterus (Duerr et al., 1999; Sze et al., 2000). However, *osm-9* and *ocr-2* mutant animals do not accumulate excess eggs in the uterus and their pharyngeal pumping rates are equivalent to wild-type animals (Fig. 4B,C). Thus, 5HT signals from the other neurons are sufficient for these behaviors. But, we cannot exclude subtle behavioral changes that are difficult to detect visually.

Discussion

The genetic evidence presented here has two major implications. The phenotype of the TRPV mutants represents exquisite specificity in the control of 5HT production, as exemplified by our demonstration that mutations of the *osm-9* and *ocr-2* TRPV genes specifically downregulate 5HT biosynthesis in the ADF neurons, and this TRPV ion channel regulation of 5HT production is mediated by a neuron-specific transcription program. Our finding that the *osm-9* and *ocr-2* TRPV channel genes act in the ADF neurons, function upstream of CaMKII to control the key 5HT biosynthesis gene *tph-1* provides insights in elucidating the genetic pathway by which a serotonergic neuron couples the activity at the cell surface and 5HT signaling.

A TRPV channel-dependent transcription program controls 5HT signaling

It has been demonstrated in many experimental systems that sensory stimuli induce 5HT signals to produce changes in behavior and physiology (e.g. Barzilai et al., 1989; Boadle-Biber, 1993; Milner et al., 1998). Until this study, no endogenous membrane protein has been shown to act in a serotonergic neuron to regulate 5HT signaling. One important finding from this study is the pronounced effect of *osm-9* and *ocr-2* mutations on the expression of the 5HT synthesis gene *tph-1* (Fig. 1). This indicates that the production of 5HT is a site where sensory information is integrated to 5HT signaling. 5HT can be released by controlled exocytosis at the synapses as well as via paracrine ‘volume transmission’, and even during the controlled exocytosis it is newly synthesized 5HT preferentially released to induce changes in the postsynaptic targets (Attwell et al., 1993; Sanders-Bush, 1982). Hence, the level of 5HT production is one mechanism controlling both forms of 5HT neurotransmission.

Transcriptional regulation may represent a general principle of regulation of hormones and neuromodulators. For example, *C. elegans*' favorite growth environment upregulates the expression of the *daf-7*/TGFβ and *daf-28*/insulin genes to induce *C. elegans* proceeding reproductive development (Schackwitz et al., 1998; Li et al., 2003); in rats, noxious sound stimuli may alter the transcription of their tryptophan hydroxylase gene in a neuron-specific manner (reviewed by Boadle-Biber, 1993); and the expression of tyrosine hydroxylase can be modulated by hormones in mice (Kumer and Vrana, 1996). Transcriptional regulation of these signaling molecules is likely a mechanism to exert a relatively slow but profound effect in the signaling pathways.

Mechanisms of TRPV channel action in the ADF neurons

Our genetic results indicate that the *osm-9* and *ocr-2* channel proteins interact with different signaling transduction pathways to induce different behavioral outputs. *osm-9* and *ocr-2* are co-expressed in four pairs of the amphid sensory neurons, and are required for a normal response to attractive and aversive odorants mediated respectively by AWA, and ASH and ADL, as well as for ASH-mediated mechanosensory and osmosensory function (Colbert et al., 1997). There are genetic evidences indicating that *osm-9* and *ocr-2* function in these sensory behaviors requires the *odr-3* Gα protein (Roayaie et al., 1998). However, *tph-1* expression is unaffected by mutations in *odr-3* or in other two Gα protein expressed in the ADF neurons (Fig. 3B). Although the exact mode of activation of OSM-9 and OCR-2 in any neuron has not yet been defined, our data indicate that the activity of the channels in the ADF neurons is regulated by different signaling molecules. It is interesting to note that *osm-9* and *ocr-2* also act in the ASH and ADL neurons to regulate social behavior independent of *odr-3* activity (de Mono et al., 2002); hence, the OSM-9 and OCR-2 can induce specific behaviors by coupling distinct signaling systems.

The reciprocal effects of the *unc-43* CaMKII loss- and gain-of-function mutations on *tph-1* expression indicate that the amount of Ca^{2+} modulates 5HT biosynthesis (Fig. 3B). The TRP superfamily is Ca^{2+}-permeable channels, and CaMKII is known as an important mediator of Ca^{2+} signaling (reviewed by Hanson and Schulman, 1992). Our genetic study shows that the constitutively active, Ca^{2+}-independent *unc-43(n498)* CaMKII (Reiner et al., 1999) can partially activate *tph-1* expression in *osm-9* deletion mutant animals (Fig. 3B). These results support the model that UNC-43 is a downstream effector of the OSM-9 and OCR-2 channels: Ca^{2+} influx through OSM-9 and OCR-2 activates UNC-43 CaMKII, which induces phosphorylation cascades to activate *tph-1* expression. However, *unc-43(lf)* mutants still express a substantial amount of *tph-1*, and the *unc-43(gf)* mutation does not completely bypass OSM-9 activity, indicating that other OSM-9/OCR-2 downstream signaling molecules may act in parallel with UNC-43 to regulate *tph-1* expression.

The effect of OSM-9 and OCR-2 TRPV ion channels in the ADF neurons is strikingly specific, given the involvement of Ca^{2+} and CaMKII. The general architecture of the ADF neurons is unaffected, we could not detect a significant change in the expression levels of ADF marker genes or genes directly involved in the serotonergic phenotype, nor we could detect an effect of *unc-43(lf)* or (gf) mutations on *tph-1* expression in other serotonergic neurons. This specificity demonstrates that 'multi-functional, widespread' signaling molecules may play a refined role in a particular native cellular setting. It is conceivable that such differential regulation of the serotonergic phenotype genes would allow the ADF neurons to adjust 5HT neurotransmission in response to multiple sensory signals. As TRPV channels are expressed in the serotonergic locus in mammals (Tominaga et al., 1998; Mezey et al., 2000), it would be interesting to determine whether there is a link between TRPV mutations and 5HT deficiency in human.

The role of TRPV channels in a sensory-neuroendocrine signaling pathway

The Dauer phenotype of the *osm-9* and *ocr-2* mutants demonstrates a genetic link between the TRPV ion channels and endocrine signaling (Fig. 4). *C. elegans* Dauer/non-Dauer development reflects two alternative metabolic states controlled by sensory inputs to neuroendocrine signaling pathways. The pathways from DAF-7/TGFβ and DAF-2/insulin receptor converge to stimulate reproductive growth; harsh environmental conditions transduced by the amphid chemosensory neurons suppress the endocrine signaling to induce Dauer arrest (reviewed by Riddle, 1997). Our enhancement and suppression genetics implies that *osm-9* and *ocr-2* regulate the DAF-2/insulin-receptor signaling pathway (Fig. 4). The TRPV channels are probably acting in the ADF neurons to modulate endocrine activity: worms bearing defective ADF neurons tend to form Dauers (Shakir et al., 1993), and laser ablation experiments implicate ADF but not the other *osm-9* and *ocr-2* co-expressing chemosensory neurons in Dauer formation (Bargmann and Horvitz, 1991b). However, expression of the wild-type *osm-9*-coding sequence under a *lin-11* promoter only partially suppresses *daf-7;osm-9* Dauer phenotype, indicating that *osm-9* activity in other cells also modulates Dauer phenotype. Alternatively, the *lin-11* promoter may not be able to express sufficient amount of *osm-9* to induce a wild-type level of ADF 5HT signals (Table 2), or ectopic expression of *osm-9* in other *lin-11*-expressing cells may interfere with the neuroendocrine signaling cascades for normal development. In mammals, 5HT regulates insulin synthesis, release and response (Breum et al., 1995; Peschke et al., 1997). It has been proposed that a feedback regulatory loop between hypothalamus 5HT and circulating hormones such as insulin, leptin and adipose tissue-derived hormone modulates the satiety and maintains metabolic and energy homeostasis (Leibowitz and Alexander, 1998). Interestingly, mouse TRPV-like channels can be activated by insulin-like growth factors (Kanzaki et al., 1999). We propose that 5HT is one mediator of TRPV channels and endocrine activity.

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