Both insulin and calcium channel signaling are required for developmental regulation of serotonin synthesis in the chemosensory ADF neurons of *Caenorhabditis elegans*

Annette O. Estevez a, Robin H. Cowie a, Kathy L. Gardner a,b, Miguel Estevez a,b,*

a Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15260, USA
b Veterans Administration Hospital, Pittsburgh, PA 15240, USA

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Abstract

Proper calcium channel and insulin signaling are essential for normal brain development. *Leaner* mice with a mutation in the P/Q-type voltage-gated calcium channel, Cacna1a, develop cerebellar atrophy and mutations in the homologous human gene are associated with increased migraine and seizure tendency. Similarly, abnormalities in insulin signaling are associated with abnormal brain growth and migraine tendency. Previously, we have shown that in the ADF chemosensory neurons of *Caenorhabditis elegans* UNC-2/Ca2+ channel function affects TGF-β-dependent developmental regulation of tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis. Here we show that developmental expression of a tryptophan hydroxylase:GFP reporter construct is similarly decreased by reduction-of-function mutations in the daf-2/insulin receptor. This decreased expression of tryptophan hydroxylase observed in both the daf-2 and unc-2 mutant backgrounds is suppressible either genetically by reduction-of-function mutations in the daf-16/forkhead transcription factor, an effector of the DAF-2/insulin receptor, or pharmacologically by the serotonin receptor antagonist cyproheptadine. Overall, these data suggest that both UNC-2 and DAF-2 function are required in the developmental regulation of DAF-16 and serotonin-dependent inhibition of tryptophan hydroxylase expression.

Introduction

Many divergent signaling pathways must be precisely coordinated and integrated with each other during normal development of the central nervous system. Mutations in genes regulating Ca2+ influx and glucose metabolism have been shown to affect both the architecture and function of the brain. In the *leeaner* mouse, a reduction-of-function (rf) mutation in the voltage-gated α1A calcium channel subunit, Cacna1a, leads to severe ataxia, absence-like seizures, cerebellar atrophy (Fletcher et al., 1996), and an increased resistance to the phenomena of cortical spreading depression (Ayata et al., 2000) that has been observed indirectly during spontaneous migraine in humans (Woods et al., 1994). A rare autosomal dominant form of migraine with severe aura, familial hemiplegic migraine (Ophoff et al., 1996), is associated with seizure and ataxia phenotypes in some families (Terwindt et al., 1997, 1998; Vanmolkot et al., 2003) and is linked to mutations in the human CACNA1A gene. Mice transgenic or mutant for insulin-like growth factor-I (IGF-I) show abnormal brain development (D’Ercole et al., 2002), whereas polymorphisms in the human insulin receptor (INSR) have been associated with common forms of migraine (McCarthy et al., 2001). Members of the forkhead transcription factor family have previously been shown to function downstream of insulin signaling in both invertebrates (Ogg et al., 1997) and humans (Schinner et al., 2005), and in a recently reported case a deletion of the FOXC1 forkhead gene cluster was shown to be associated with numerous brain abnormalities including hydrocephalus and
hypoplasia of the cerebellum, the brain stem, and the corpus callosum (Maclean et al., 2005). The identification of these human and mouse genetic variants with phenotypes exhibiting overlapping developmental effects raises the question of whether they might affect a common signaling pathway.

The free living soil nematode, Caenorhabditis elegans, has been used extensively to unravel the complexities of signaling mechanisms due in part to its simple body plan (approximately 300 neurons in an adult animal) and short life cycle (2 weeks at ambient temperatures). Similar to the mouse, mutations in unc-2, the C. elegans ortholog of CACNA1A, cause the animals to display uncoordinated ataxic-like movement (Brenner, 1974) and seizure-like activity (M. Estevez, unpublished). Additionally, unc-2 animals are hypersensitive to serotonin (Schafer and Kenyon, 1995), a monoaminergic neurotransmitter that is critical for normal vertebrate brain development, as well as post-developmental remodeling and maintenance of neuronal tone (Gaspar et al., 2003; Sawin et al., 2000; Hardaker et al., 2001; Estevez et al., 2004) and is important for normal neuronal migration in the developing nervous system (Kindt et al., 2002). The rate-limiting enzyme for serotonin synthesis, tryptophan hydroxylase (encoded by tph-1), is expressed in nine neurons (Sze et al., 2000) including ADF, a bilaterally symmetric pair of chemosensory neurons with sensory processes directly exposed to the environment. Recently, we have shown (Estevez et al., 2004) that tph-1 expression in the ADF neurons is regulated by UNC-2 through the activities of a transforming growth factor (TGF)-β signaling pathway regulating growth and developmental arrest.

Under stressful environmental conditions when low levels of food and high levels of a constitutively secreted pheromone are detected, C. elegans can metabolically and developmentally arrest as dauer larva, an alternative third larval stage from which animals can recover to re-enter normal development (Cassada and Russell, 1975; reviewed in Riddle, 1988). Constitutive dauer larva formation (Daf-C) can also be induced by laser ablation of the ADF and ASI neurons (Bargmann and Horvitz, 1991) or at high temperatures (25°C) by ts mutations in genes encoding members of either the DAF-7/TGF-β or DAF-2/insulin-like growth factor pathways (reviewed in Riddle, 1988). Additionally, a mutation in tph-1 has been reported to cause some animals to constitutively arrest as dauer larva due in part to the coordinate downregulation of both the TGF-β and insulin-like growth factor pathways (Sze et al., 2000). Because our previous data showed that the UNC-2-regulated TGF-β signaling cascade affects the expression of tph-1, we set out to determine whether insulin signaling also contributes to the overall regulation of serotonin synthesis. The findings presented here suggest that the DAF-2/insulin signaling pathway is involved in the control of serotonin synthesis in the ADF neurons, and that along with the UNC-2-regulated TGF-β signaling pathway modulates tph-1 expression by regulating the activities of the DAF-16/Forkhead transcription factor.

Materials and methods

Maintenance and growth of strains

Animals were maintained and grown at 25°C on modified (without added Ca++) nematode growth medium (NGM) under standard conditions (Brenner, 1974). Dauer larvae were either induced to form at high temperature (25°C) due to the temperature sensitive (ts) nature of the mutations in the daf genes studied, or as for the WT and unc-2(ts) animals, by starvation.

The following mutations were used: L1, daf-16(m26, mgDf50); LGIII, daf-2(e1368, e1370, m41), daf-4(m592); LGIV, him-8(e1489), unc-43(e408); LGV, akb-1(mg144); LGX, daf-3(e1576), pdk-1(mg142, sa709), unc-2(e35). The GR1333 strain that contains the integrated transgenes tph-1::GFP (Sze et al., 2000) and pRF4 (a plasmid expressing a dominant rol-6 mutation that causes animals to roll) was used to construct all the strains used in the analysis of tph-1 expression.

Stereomicroscopy and scoring of GFP expression

Four to six young adult hermaphrodites were placed on each of three to five plates and the ADF phenotype of their progeny was visualized using a Leica MZFLIII stereomicroscope equipped with fluorescence optics (Leica Microsystems Inc., Bannockburn, IL). An average of 100 adult or dauer larvae progeny were counted from each plate (total number of animals counted per strain is listed as the n value in Supplementary Data Tables 1–3). Animals were considered to display the ADF-S phenotype if the expression of the tph-1::GFP reporter in their ADF neurons was of similar intensity to that observed in their NSM neurons, as described (Estevez et al., 2004). The symmetry/asymmetry phenotype scored in Fig. 3F was not dependent on the phenotype of expression in the NSM neurons and was scored for any expression, weak or strong, that could be detected in either of the ADF neurons: expression in both neurons was scored as “symmetric”; in one neuron as “asymmetric”; and in neither neuron as “none”. The ADF phenotype of individual animals was documented by visualization on a Leica DM IRB microscope equipped with fluorescence optics and a DC300F imaging system (Leica Microsystems Inc., Bannockburn, IL), and the image was processed for publication using the Adobe Creative Suite software (Adobe Systems Inc., San Jose, CA). In order to ascertain the effects of the serotonin antagonist cyproheptadine on the ADF phenotype, animals were grown from the egg to the adult or dauer larvae stage on plates supplemented to a final concentration of 100 μM cyproheptadine (Sigma, St. Louis, MO, USA) diluted in dH2O.

Adult animals that developed at 25°C even though they carried a ts mutation in a daf-2 gene did so by either avoiding entering or spontaneously recovering from the dauer larval stage. To determine which paradigm was most likely, we tested recovered dauer larva for both their ADF phenotype and their ability to spontaneously recover at 25°C. Worms grown at high temperature (25°C) were washed from their plates and treated with a solution of 1% SDS as described (Swanson and Riddle, 1981; Mains and McGhee, 1999) to isolate dauer larvae. The dauer larvae were either recovered at 15°C overnight then returned to grow at 25°C, or were directly placed at 25°C. The ADF-S phenotype of the SDS dauer larvae recovered at 15°C was scored (as described above) two days after treatment. Because less than 2% of the SDS dauer larvae placed directly at 25°C recovered, their phenotype was not scored. Hence, on the basis of comparison, these two results together strongly suggest that the ADF-S phenotype of the daf-2 adults scored at 25°C was in fact derived from animals that avoided larval arrest.

Statistical analysis was performed using Microsoft Excel software (Microsoft Corporation). The means and standard deviations (SD) reported were determined by averaging data obtained from all the plates of each strain or population type (e.g., treated or untreated) counted. P values were determined by applying a two-tailed Student’s t test with unequal variance. Comparisons were defined as significant if they were determined to have a p value less than 0.05. Graphs were initially drawn using Excel and were prepared for publication using Adobe Creative Suites (Adobe Systems Inc., San Jose, CA).
Results

DAF-2 and UNC-2 coordinately regulate neuron-specific tryptophan hydroxylase expression and serotonergic signaling during larval arrest

When animals are grown at 25°C, the expression of a GFP reporter under the control of the tph-1 promoter (Sze et al., 2000) is of approximately equal intensity in two head neurons, NSM and ADF (we have defined this phenotype as ADF-S) within roughly 80% of wild-type (WT) animals, whereas a reduction-of-function (rf) mutation in the unc-2 gene results in a 30-fold decrease in the percentage of animals with strong expression in ADF (defined as ADF-W) but no apparent change in NSM expression (Estevez et al., 2004). We have shown previously (Estevez et al., 2004) that when grown at high temperature (25°C), the percentage of unc-2(rf) animals expressing the ADF-S phenotype could be dramatically increased to near WT levels by passage through the dauer larva stage, whether induced by starvation or by the addition of a rf mutation in either daf-2 (Ren et al., 1996) or daf-4 (Estevez et al., 1993), two members of a TGF-β signaling pathway involved in regulating this developmental decision. Because the DAF-2/insulin pathway functions in parallel to this TGF-β pathway in controlling dauer larva development, we investigated whether the DAF-2/insulin pathway also affects the ADF-S phenotype of unc-2.

The daf-2(rf) mutations have been previously characterized (Gems et al., 1998) as either class 1 or class 2 alleles based on a number of phenotypic criteria including Daf-C, intrinsic thermotolerance (Itt), and life span extension (Age). Of the class 1 mutations that have thus far been identified, all of them have been shown to map within the region of daf-2 that encodes its ligand binding domain whereas the class 2 mutations as a whole fall within the region encoding the DAF-2 kinase domain (Kimura et al., 1997). We examined three daf-2(rf) alleles for their effects on the ADF-S phenotype: two class 1, e1368 and m41; and one class 2, e1370. All three alleles produced populations of dauer larvae with percentages of ADF-S animals between 45% and 54% (Fig. 1F; Supplementary Table 1), which is significantly lower (p < 0.05; compare Figs. 1B and C with Fig. 1D; Fig. 1F; and Supplementary Table 1) than that observed in populations of WT dauer larva that were 100% for the ADF-S phenotype (Fig. 1F; Supplementary Table 1). Given that this reduction was observed in all three daf-2 alleles, regardless of class type, this suggests that DAF-2/insulin receptor signaling integrates into the developmental regulation of tph-1 expression during dauer larval arrest.

As mentioned above, the ADF phenotype of unc-2(rf) dauer larvae is near that of WT (p = 0.08; Figs. 1C and F; Supplementary Table 1), which is in contrast to the reduced expression levels observed in dauer larvae of all three daf-2 alleles. In order to determine whether a relationship exists between UNC-2/calcium channel and DAF-2/insulin receptor signaling to regulate tph-1 expression during dauer larval arrest, we constructed strains containing both the unc-2(e55) mutation and one of each of the three daf-2 alleles: e1368, e1370, or m41.

All three daf-2;unc-2 double mutant strains were significantly lower than their unc-2(e55) parent strain (p < 0.05; Fig. 1F; Supplementary Table 1), whereas comparisons of the daf-2;unc-2 doubles to their single daf-2 parents showed allele-specific differences. Neither strain containing a class 1 daf-2 mutation, e1368;unc-2, nor m41;unc-2 was different from its parent daf-2 strain (e1368; p = 0.37, and m41; p = 0.62, respectively; Figs. 1D–F; Supplementary Table 1). In contrast, the percentage of ADF-S animals in the class 2 e1370;unc-2 strain was improved (p = 0.03; Fig. 1F; Supplementary Table 1), increasing to 86% (SD ± 4.0%) the percentage of ADF-S animals from a baseline of 54% (SD ± 12.1%) for the daf-2(e1370) parent strain (Fig. 1F; Supplementary Table 1). The observed differences in the effects of the daf-2 alleles on the ADF-S phenotype of the unc-2 dauer larvae suggest a complex interaction between DAF-2 and UNC-2 signaling in the regulation of tph-1 expression during dauer larval development.

Modulation of the postsynaptic response to serotonin has been suggested to require the activation of the UNC-2/calcium channel (Schafer and Kenyon, 1995). This association between serotonin and UNC-2 is further supported by the observation that growing WT animals in exogenous serotonin results in transient lethargic movement whereas in unc-2(rf) exogenous serotonin results in persistent lethargy (Schafer and Kenyon, 1995). These observations show that desensitization to serotonin requires UNC-2 function and suggests the possibility that in the absence of UNC-2 function serotonin signaling may be increased at baseline. Consistent with this, we previously reported (Estevez et al., 2004) that in the unc-2(rf) background serotonin receptor (5HT2) blockade with cyproheptadine improves movement and may decrease basal serotonergic signaling that reduces expression of tph-1 in ADF relative to WT. It was suggested by Zhang et al. (2004) that 5HT in the ADF neurons may be partially involved in regulating dauer larva formation when induced by Daf-C mutations in the DAF-2/insulin signaling pathway. To investigate whether 5HT is also involved in regulating the expression of the tph-1 transgene in dauer larva and whether this involves either or both DAF-2 and UNC-2 signaling, we grew the parent and daf-2;unc-2 double mutant strains in a final concentration of 100 μM cyproheptadine, a serotonin receptor antagonist, as previously described (Estevez et al., 2004). Although when treated with cyproheptadine, the percentage of ADF-S daf-2(e1368) dauer larvae was increased two-fold over the untreated control (p = 1.15 × 10⁻³; Fig. 1F; Supplementary Table 1), it still remained significantly less in comparison to both the treated WT and unc-2 dauer larvae (p = 3.76 × 10⁻³ and 1.59 × 10⁻³, respectively; Fig. 1F; Supplementary Table 1). No increase was observed after treatment of either the daf-2(m41) or the daf-2(e1370) dauer larvae (p = 0.33 and 0.31, respectively; Fig. 1F; Supplementary Table 1). Because all three alleles of daf-2 tested showed an attenuated response to cyproheptadine, it is likely that both 5HT and DAF-2 signaling are required for regulation of tph-1 expression in the ADF neurons of dauer larvae. Although the lack of responsiveness to cyproheptadine observed in both the daf-2(m41);unc-2 and daf-2(e1370);unc-2 dauer larvae was not surprising because neither of their single daf-2 parent strains
responded significantly to cyproheptadine treatment ($p > 0.05$; Fig. 1F; Supplementary Table 1), the similar unresponsiveness of the $daf-2(e1368); unc-2$ strain ($p = 0.34$; Fig. 1F; Supplementary Table 1) represents a phenotype distinct from its $daf-2(e1368)$ parent strain ($p = 2.97 \times 10^{-2}$; Fig. 1F; Supplementary Table 1), which showed an increased percentage of ADF-S expression in response to cyproheptadine treatment. This attenuated response of the $daf-2(e1368); unc-2$ double is different from either parent and may reflect that there is some degree of functional overlap in the DAF-2 and UNC-2 signaling that interacts with the serotonergic pathway to control $tph-1$ expression in dauer larvae.

**DAF-2 requires DAF-16 to regulate tryptophan hydroxylase expression**

RF mutations in the DAF-16/Forkhead/winged-helix transcription factor antagonize the life extension (Lin et al., 1997)
and constitutive larval arrest phenotypes induced by rf mutations in \textit{daf-2} (Gottlieb and Ruvkun, 1994; Lee et al., 2001). Because \textit{daf-16}(rf) animals do not form dauer larva (they have a dauer larva formation defective phenotype, Daf-D), and growing them in dauer inducing pheromone or adding a \textit{daf-7}/TGF-\beta rf mutation induces only partial dauer formation (Ogg et al., 1997), we found it necessary to study adult animals to determine the effects of \textit{daf-16} mutations on the regulation of \textit{tph-1} expression. The \textit{tph-1}:\textit{GFP} transgene is only weakly expressed in ADF at 15\textdegree{}C in WT (Estevez et al., 2004) and all of the \textit{daf-2} and \textit{daf-16} strains examined (<10\%; data not shown); therefore, the ADF-S phenotype was scored at 25\textdegree{}C. Although when grown at 25\textdegree{}C the \textit{daf-2} alleles produce mostly dauer larva, some of the \textit{e1368} and \textit{m41} alleles developed to adulthood allowing these two strains to be assayed at their restrictive temperature. These 25\textdegree{}C grown adults most likely escaped larval arrest because the ADF-S phenotype of \textit{daf-2}(m41);\textit{unc-2} adults derived from recovered constitutive dauer larvae (55.33 \pm 3.21\%; Supplementary Table 2) was statistically indistinguishable (\(p = 0.32\); Supplementary Table 2) from the ADF-S phenotype observed for the dauer larvae (Fig. 1F; Supplementary Table 1), but significantly different (\(p = 8.39 \times 10^{-4}\); Supplementary Table 2) than that of the adult population that presumably did not pass through the dauer larval stage (Fig. 2G; Supplementary Table 2).

UNC-2 signaling is essential for regulating \textit{tph-1} expression in ADF, based on the low expression levels previously reported for \textit{unc-2}(rf) adults grown at 25\textdegree{}C (Estevez et al., 2004; and shown here, Figs. 2B and G; Supplementary Table 2). The percentages of ADF-\textit{S daf-2(e1368)} (Figs. 2C and G; Supplementary Table 2) and \textit{daf-2(m41)} (Fig. 2G; Supplementary Table 2) animals were like that of the \textit{unc-2}(rf) adults (\(p > 0.05\); Fig. 2G; Supplementary Table 2) and were significantly lower than WT (\(p < 0.05\); Figs. 2A and G). This suggests that the temperature-dependent ADF-S phenotype seen in adult animals similarly requires DAF-2 signaling, as was observed in the dauer larvae populations (Fig. 1). The observations recorded for the adult \textit{daf-2(e1368);unc-2} and \textit{daf-2(m41);unc-2} animals also paralleled those from the dauer larvae in that the addition of the \textit{unc-2}(rf) mutation did not result in increased percentages of ADF-S expression (Figs. 2D and G; Supplementary Table 2). Yet, the results differed from those of the dauer larvae in that the expression levels observed for the \textit{daf-2(m41);unc-2} animals were lower than that of either its \textit{unc-2} or \textit{daf-2} parent (Fig. 2G; Supplementary Table 2). Based on the combined results from both the dauer larva and adult populations, it is plausible to suggest that the differences observed for the \textit{daf-2} alleles in the \textit{unc-2} background represent an allelic severity series with regard to expression of \textit{tph-1} in the ADF neurons, as has been described for other \textit{daf-2} phenotypes (Gems et al., 1998). For example, the \textit{m41} allele is one of the weakest alleles for the ITT and Age phenotypes but has one of the strongest Daf-C phenotypes at 25\textdegree{}C. Given the additive effects of the \textit{m41} allele on the ADF-W phenotype, we suggest that this most likely represents the more severe of the three alleles studied in regards to the regulation of expression of \textit{tph-1} in the \textit{unc-2}(rf) background.

As reported above, of the three \textit{daf-2} alleles tested only the \textit{daf-2(e1368)} dauer larvae responded to serotonergic blockade prompting us to similarly test their adult progeny. Growth on cyproheptadine resulted in a dramatic increase in the percentage of ADF-\textit{S daf-2(e1368)} adult animals observed when compared to the untreated \textit{daf-2(e1368)} parent strain (\(p = 3.6 \times 10^{-7}\); Fig. 2G; Supplementary Table 2), which was comparable to the results observed in the dauer larva but differed because they recovered fully to WT levels (\(p = 0.10\); Fig. 2G; Supplementary Table 2). Treatment of the \textit{unc-2}(rf) adults with cyproheptadine also resulted in WT-like expression levels (\(p = 0.12\)). The increases observed with treatment of both single mutant parent strains were partially blocked in the \textit{daf-2(e1368);unc-2} double mutant strain, which had a frequency of ADF-S expression that was 40\% less than either parent strain in response to cyproheptadine (\(p < 10^{-3}\); Fig. 2G; Supplementary Table 2). This attenuated response to cyproheptadine treatment is similar to that observed for the \textit{daf-2(e1368);unc-2} dauer larvae, which did not respond to serotonergic blockade (Fig. 1F; Supplementary Table 1). These findings are again consistent with DAF-2 and UNC-2 having an overlapping role in signaling that inhibits serotonergic downregulation of \textit{tph-1} expression in the ADF neurons.

Two major isoforms of DAF-16, DAF-16a and DAF-16b, have distinct DNA binding domains (Ogg et al., 1997). We tested two alleles of \textit{daf-16}, \textit{m26} which is specific to the DAF-16a isoform and \textit{mgDf50} which deletes both isoforms (Ogg et al., 1997), for their ability to improve the weak ADF expression observed in the single and double mutant combinations of \textit{daf-2} and \textit{unc-2}. The observed percentages of ADF-S animals in the \textit{m26;unc-2} double and \textit{m26;daf-2;unc-2} triple mutant strains were greater than WT (94–100\%, compared to 90\% for WT; Fig. 2G; Supplementary Table 2), whereas the \textit{mgDf50;unc-2} and \textit{mgDf50;daf-2;unc-2} strains were significantly better than their single and double parent strains, but had a reduced frequency (51–85\%; Fig. 2G; Supplementary Table 2) compared to WT (90\%; Fig. 2G; Supplementary Table 2). This relatively reduced ability of the \textit{daf-16(mgDf50)} allele to increase \textit{tph-1} expression in both the \textit{unc-2} and \textit{daf-2;unc-2} backgrounds is curious, but one possible explanation for this unanticipated result is that the two isoforms of DAF-16 that have distinct amino acid sequences in their DNA binding domains (Ogg et al., 1997) may bind separate regions of the \textit{tph-1} promoter having opposite effects on the expression of \textit{tph-1}. In this scenario, the \textit{m26} allele that is specific to DAF-16a deletes the isoform that has an inhibitory affect on the expression of \textit{tph-1} in ADF, leaving intact the DAF-16b isoform, which positively regulates the \textit{tph-1} promoter leading to an observed increase in transgene expression. The \textit{mgDf50} allele that deletes both isoforms removes both the negative and positive control of \textit{tph-1} expression in ADF, allowing baseline \textit{tph-1} expression to be observed. This “baseline” expression may be ubiquitous or may be due to regulation by an additional modulator that is independent of the insulin signaling pathway.

Both PDK-1 and AKT-1 have been shown to function downstream of the DAF-2 receptor, but upstream of the DAF-16 transcription factor affecting both lifespan and dauer larval development (Paradis and Ruvkun, 1998; Paradis et al., 1999;
To determine if these two kinases function to regulate tph-1 expression, we constructed strains containing mutations in either \textit{pdk-1} or \textit{akt-1} and the \textit{tph-1} transgene. As expected, an \textit{rf} mutation in \textit{pdk-1} had a reduced frequency of adult animals with ADF-S expression which increased in response to cyproheptadine treatment similar to the \textit{daf-2} \textit{e1368} and \textit{m41} \textit{rf} alleles of \textit{daf-2} predominantly exhibited the ADF-W phenotype. The prevalence of the ADF-W phenotype was unchanged in the \textit{daf-2} \textit{m41};\textit{unc-2} \textit{G} strain, where less animals had the ADF-S phenotype than either its \textit{daf-2} or the \textit{unc-2} single mutant parent strain. This enhanced ADF-W phenotype suggests that the DAF-2 and UNC-2 signaling pathways both interact in the regulation of \textit{tph-1} expression in ADF. Two alleles of \textit{daf-16}, \textit{m26} (E-G), and \textit{mDf50} (G) were able to increase the ADF expression levels for both the \textit{daf-16};\textit{unc-2} (E and G) and \textit{daf-16};\textit{unc-2} (F and G) strains. Phosphorylation by the kinases PDK-1 and AKT-1 have been shown to prevent nuclear localization of DAF-16 (reviewed by Nelson and Padgett, 2003). An \textit{rf} mutation in \textit{pdk-1} exhibited a lower frequency of ADF-S animals whereas gain-of-function (gf) mutations in both \textit{pdk-1} and \textit{akt-1} were able to increase the percentage of ADF-S animals in an \textit{unc-2} background (G). Cyproheptadine treatment increased the percentage of animals with an ADF-S phenotype to near WT levels for all adult animals tested except the \textit{daf-2} \textit{m41};\textit{unc-2} double mutant strain, which increased to within only 40% of the treated WT animals (G), suggesting that DAF-2 and UNC-2 may have partially non-redundant roles in the regulation of \textit{tph-1} expression in the ADF neurons. All symbols and abbreviations were as in Fig. 1 with the addition of \textit{d16}, \textit{daf-16} (allele as listed); \textit{pdk-1} \textit{rf}; \textit{pdk-1} \textit{sa709}; \textit{pdk-1} \textit{gf}; \textit{pdk-1} \textit{mg142}; \textit{akt-1} \textit{gf}; \textit{akt-1} \textit{mg144}. Black bars and gray bars represent the percentage of animals untreated or treated (respectively) with 100 \textmu M cyproheptadine whereas strains in which the response to cyproheptadine was not determined are marked with ND.
DAF-16 is required for regulation of tph-1 by the TGF-β signaling pathway

An association between the daf-7/TGF-β pathway and tph-1 has been suggested previously by studies showing that the tph-1 mutation decreased expression of a daf-7::GFP reporter construct while enhancing the DAF-C phenotype of daf-7(9f) animals (Sze et al., 2000). In contrast, we have shown that in an unc-2(9f) background, rf mutations in either the gene encoding the DAF-7 ligand or the DAF-4 receptor increased the expression of the tph-1::GFP transgene, indicating the involvement of the DAF-7/TGF-β pathway in the UNC-2-dependent regulation of tph-1 expression itself (Estevez et al., 2004). UNC-2-dependent Ca2+ influx has also been hypothesized to be linked to UNC-43/CaMKII (Reiner et al., 1999) regulation that affects the symmetry of neuronal gene expression (Troemel et al., 1999) and neuronal migration (Tam et al., 2000), as well as tph-1 upregulation (Estevez et al., 2004).

Because the DAF-16 transcription factor appears to function downstream of both DAF-2 and UNC-2 to regulate the TGF-β and CaMKII pathways. This lack of responsiveness to cyproheptadine was also observed in the unc-2(9f) strain, which was increased to near WT levels in a dauer larva development (Ren et al., 1996) or decreased serotonin synthesis (Sze et al., 2000). Our studies presented here suggest that the DAF-2/insulin signaling pathway is also involved in the regulation of tryptophan hydroxylase expression in the chemosensory ADF neurons, and that this regulation involves the coordinate control of the DAF-16/forkhead transcription factor by both the DAF-2/insulin and UNC-2-regulated TGF-β signaling pathways. Additionally, the pattern of asymmetric expression and reduced levels of tph-1 expression observed in the ADF neurons of the strains containing the ADF-S phenotype, and as expected when comparing rf with gf, were consistent with its effect on the ADF-S phenotype, and as expected when comparing rf with gf, opposite to that observed for the unc-43(gf) allele’s affect on the symmetry of serotonin expression (Estevez et al., 2004). The daf-16(m26) allele was capable of suppressing the asymmetric expression of both the unc-2 and unc-43,unc-2 strains (Fig. 3F). If UNC-43 functions in the DAF-2/insulin signaling pathway as implied by the above data, then these results suggest that DAF-16 acts downstream of both this pathway and the DAF-7/TGF-β pathway to regulate both the pattern and level of expression of tph-1 in the ADF neurons.

Discussion

Previously, we have reported (Estevez et al., 2004) that expression of a tph-1::GFP transgene in the ADF chemosensory neurons of well-fed populations of unc-2(9f) animals is decreased relative to similarly fed WT populations, and that this unc-2(9f) phenotype could be suppressed by starvation-induced dauer larva development, reduction-of-function mutations in Daf-C-type TGF-β signaling genes, or serotonin receptor blockade with the 5HT2 receptor antagonist, cyproheptadine. These findings are all consistent with the observations of other researchers showing that the expression of the gene encoding the TGF-β ligand, DAF-7, is reduced by starvation-induced dauer larva development (Ren et al., 1996) or decreased serotonin synthesis (Sze et al., 2000). Our studies presented here suggest that the DAF-2/insulin signaling pathway is also involved in the regulation of tryptophan hydroxylase expression in the chemosensory ADF neurons, and that this regulation involves the coordinate control of the DAF-16/forkhead transcription factor by both the DAF-2/insulin and UNC-2-regulated TGF-β signaling pathways. Additionally, the pattern of asymmetric expression and reduced levels of tph-1 expression observed in the ADF neurons of the strains containing the ADF-S phenotype, and as expected when comparing rf with gf, were consistent with its effect on the ADF-S phenotype, and as expected when comparing rf with gf, opposite to that observed for the unc-43(gf) allele’s affect on the symmetry of serotonin expression (Estevez et al., 2004). The daf-16(m26) allele was capable of suppressing the asymmetric expression of both the unc-2 and unc-43,unc-2 strains (Fig. 3F). If UNC-43 functions in the DAF-2/insulin signaling pathway as implied by the above data, then these results suggest that DAF-16 acts downstream of both this pathway and the DAF-7/TGF-β pathway to regulate both the pattern and level of expression of tph-1 in the ADF neurons.

Further evidence in support of this hypothesis comes from our studies of adult animals with rf mutations in daf-16 and daf-3, genes encoding downstream transcriptional regulators of the
DAF-2/insulin and the DAF-7/TGF-β signaling pathways, respectively. Although strains with these mutations have a similar Daf-D phenotype (i.e., disable the dauer larva developmental switch), they in fact have opposite effects on tph-1 expression in the ADF neurons (Figs. 2 and 3). One possible explanation for this is that the DAF-16 and DAF-3 proteins independently bind to distinct regions of the tph-1 gene (Fig. 5A). The ADF-W phenotype of the daf-3(rf) mutation suggests

**Fig. 3.** daf-16 appears epistatic to both the UNC-2/TGF-β and the DAF-2 signaling pathways. RF alleles of the daf-4/TGF-β receptor suppress the weak expression pattern of unc-2, increasing the number of animals with strong expression in their ADF neurons (Estevez et al., 2004). This phenotypic suppression can be reverted to unc-2 expression levels by the addition of an rf mutation in the daf-3/SMAD transcription factor (Patterson et al., 1997), a downstream effector of TGF-β signaling (A). Either treatment with cyproheptadine (E, gray bar) or the addition of a daf-16(rf) mutation (B) was capable of increasing the percentage of ADF-S daf-4;unc-2daf-3 animals (E). Although daf-16 suppressed the ADF-W phenotype observed in the unc-43(rf);unc-2 strain (C) to near WT levels (D and E), treatment with cyproheptadine did not cause an increase in expression (E). (F) Expression of the tph-1::GFP transgene is symmetrically expressed in the ADF neurons of nearly 100% of WT animals. ADF expression was not observed in >50% of unc-2 animals, a number increased to >80% in the unc-43;unc-2 strain. A pattern more similar to WT was observed in the daf-16;unc-43;unc-2 triple mutant strain. The pattern of the unc-43(rf) parent strain was identical to that of the unc-43;unc-2 strain (data not shown). The symmetry of ADF expression in panel F was not determined relative to the expression in NSM as for Figs. 1 and 2, and panel E above, but was scored as present if any expression weak or strong was observed in either ADF neuron (one = asymmetric; both = symmetric). Black and gray bars in panel E represent the percentage of animals untreated or treated (respectively) with 100 μM cyproheptadine whereas strains in which the response to cyproheptadine was not determined are marked with ND. The identities of the bars in panel F are shown. All symbols and abbreviations are as in Fig. 1 with the following additions: d3, daf-3(e1376); d4, daf-4(m592); d16, daf-16(m26); u43, unc-43(e408).
that the role of DAF-3 would be to activate transcription of tph-1 whereas that of DAF-16 would be to repress or inactivate transcription. A similar signaling paradigm has been found for another C. elegans forkhead transcription factor, PHA-4, which binds and activates a region of the pharyngeal muscle protein gene, myo-2 (Kalb et al., 1998). Binding of the DAF-3/Smad within a 28-bp region containing the PHA-4 binding site was shown to act as a repressor of pharyngeal muscle development (Thatcher et al., 1999). In support of this as a potential model for shown to act as a repressor of pharyngeal muscle development within a 28-bp region containing the PHA-4 binding site was identified by Furuyama et al. (2000) as DBEs that bound DAF-16 (Kalb et al., 1998). The consensus sequence and 4 kb upstream of the reported start site (Fig. 4A). Additionally, two potential DAF-3 binding sites were identified (Fig. 4B), but they were not near the DBE or IRE sites as was observed with the cassette of activator and repressor sites found within the myo-2 gene although this does not rule out the possibility that secondary structure in the tph-1 promoter region may bring the DBE or IRE sites within proximity of the DAF-3 binding sites giving rise to their observed combinatorial role. It has been suggested that genes controlling dauer larval development that have upstream DBE sites are more likely to be upregulated by DAF-16 than those with sites downstream (Liu et al., 2004), yet this may not be the case for regulation of tph-1. Given the high levels of expression of the tph-1::GFP transgene in the daf-16(m26) strains, the WT function of DAF-16 is presumably to inhibit tph-1 transcription. Because DAF-16 and DAF-3 appear to function in opposition to each other during regulation of tph-1 expression as opposed to their roles in dauer larva development (as noted above), it is possible that the correlation between location of the DBE sites and regulation of gene expression by DAF-16 might also be in opposition to that previously described.

Another possible model for DAF-16 and DAF-3 regulation of tph-1 is that suggested by Seoane et al. (2004) in which a forkhead transcription factor directly suppresses a SMAD protein. In their paper, they describe a model in which the forkhead transcription factor FoxG1 inhibits the activation of the growth inhibitory gene p21Cip1 by binding a SMAD transcriptional complex that includes an additional forkhead protein FoxO. Thus, in this example, one FOX protein activates transcription in a complex with a SMAD protein while another inactivates the complex and prevents transcription. As we proposed earlier in the text, it is possible that the two major isoforms of DAF-16 have opposite effects on the regulation of tph-1, functioning similarly to the FoxG1 and FoxO proteins in a complex with the DAF-3/SMAD to regulate transcription of tph-1 (Figs. 5B and C). In this case, the DAF-16b isoform may bind DAF-3 forming a complex that activates transcription (Fig. 5B) whereas the addition of the DAF-16a isoform would lead to inactivation of the complex (Fig. 5C). Loss of both isoforms, as in the daf-16(mgDf30) null mutation, may allow for low level transcriptional activity by the DAF-3/SMAD, which is normally enhanced by the DAF-16b isoform. This would be consistent with the observed differences in the levels of tph-1 expression in the two daf-16 backgrounds, but further molecular studies will be required to confirm or rule this out.

Our observation that the increased tph-1 expression seen in dauer larvae and adult daf-2(e1368) animals treated with cyproheptadine (Figs. 1F and 2G, respectively) was reduced in daf-2(e1368);unc-2(rf) double mutant populations suggests that the interaction between serotonergic signaling and the DAF-2R/insulin cascade is at least partially dependent on the function of the UNC-2 channel (Fig. 5A). Additionally, the genetic interaction between unc-2(rf) and daf-2(rf) revealed by the resistance of dauer larvae in certain allelic combinations to upregulation of tph-1 by a serotonin receptor antagonist (Fig. 1F) provides further support that the DAF-2/insulin and UNC-2-mediated TGF-β signaling pathways function together to regulate the level of tph-1 expression in ADF because at least one of the pathways must be active in certain allelic combinations to allow the drug-induced response. It further suggests that inhibitory 5HT signaling affects UNC-2 Ca2+-dependent tph-1 regulation, possibly through a G-protein-activated pathway as has been described for neuronal migration in C. elegans (Tam et al., 2000) and suggested by G-protein binding site mutations identified in human families with CACNA1A-dependent FHM (Melliti et al., 2003). The UNC-43/CaMKII is predicted to be a downstream effector of UNC-2 activation (Fig. 5A) based on several lines of evidence, including its ability to suppress the unc-2(rf) mutation’s effect on str-2 expression in the olfactory AWC neurons (Tanaka-Hino et al., 2002) and the gf allele affecting an increase in expression of tph-1 in the ADF neurons of the unc-2(rf) strain (Estevez et al., 2004). In our previous work (Estevez et al., 2004), we advanced the hypothesis that UNC-43 may function as an inhibitor of DAF-8/DAF-14/SMAD (Fig. 5A) based on the similarity of the ADF-S phenotypes of unc-43(gf), daf-8(rf), and daf-14(rf) in the unc-2(rf) background, and studies by others (Wicks et al., 2000) showing in vitro phosphorylation of SMAD by CaMKII can inhibit nuclear translocation (Fig. 5A). The fact that daf-3(rf) can partially suppress (reduced by 67%; Estevez et al., 2004) the ADF-S

![Image](45x141 to 272x258)

**Fig. 4.** The upstream region of the tph-1 promoter contains potential binding sites for DAF-16 and DAF-3. The consensus sequence for the DAF-16 binding element (DBE) is shown in bold above two potential binding sites located upstream of the tph-1 gene (A). Although the sequence is not an exact match, the variable nucleotides (indicated by bold underlines) correspond to nucleotides identified by Furuyama et al. (2000) as DBEs that bound DAF-16 in vitro. The insulin response element (IRE) shown (A) is an additional potential binding site for DAF-16 identified upstream of the tph-1 start. The consensus sequence and possible locations of DAF-3 binding elements are also shown (B).

**A**

<table>
<thead>
<tr>
<th>DBE: nttGTTTTTACann</th>
<th>IRE: TT (A/G) TTTT G</th>
</tr>
</thead>
<tbody>
<tr>
<td>tctTGtTTTGGCga</td>
<td>-134</td>
</tr>
<tr>
<td>asatGTTTTTCTat</td>
<td>-714</td>
</tr>
<tr>
<td>caatGTTTTTCTatat</td>
<td>-751</td>
</tr>
<tr>
<td>utttGTtTTTCoa</td>
<td>-1905</td>
</tr>
</tbody>
</table>

**B**

DAF-3 : GTCTG -946, -1545
phenotype of unc-43(gf);unc-2(ef) suggests that UNC-43/CaMKII can function as a parallel inhibitory input into the DAF-7/TGF-β pathway with DAF-3 as the ultimate regulated target. However, this simple model does not adequately explain the present data showing that cyproheptadine treatment results in an ADF-S phenotype in daf-4;unc-2daf-3 animals but not in unc-43(ef) or unc-43(ef);unc-2 animals (Fig. 3E), an observation implying that UNC-43/CaMKII can act independent of the DAF-7/TGF-β pathway in a 5HT inhibited signal enhancing tph-1 expression. The potential signaling outputs extending from UNC-43/CaMKII are extensive (Soderling, 1999) and include two potential targets that may suggest an explanation for

Fig. 5. Coordinate regulation of serotonin synthesis requires signaling input from both the insulin receptor and TGF-β pathways. (A) In this model, the DAF-16 and DAF-3 transcription factors responding to signaling inputs from the DAF-2/insulin receptor and DAF-7/TGF-β pathways (respectively) bind to the promoter of the tph-1 gene. As posited in the text, binding by the DAF-16/forkhead protein would inhibit transcription whereas the DAF-3/SMAD binding leads to activation of the tph-1 promoter. Nuclear translocation of the DAF-16 and DAF-3 proteins is required for these transcription factors to exert their effects on downstream targets (Patterson et al., 1997; Lee et al., 2001), which has been shown to be affected by the phosphorylation state of these transcription factors (Shi and Massague, 2003; Van Der Heide et al., 2004). Ca²⁺-dependent phosphorylation by the UNC-43/CAMKinase of upstream modulators (such as DAF-8/14 and AKT-1) may be an additional branch point for modulation of both the insulin and TGF-β signaling inputs into the regulation of tph-1 transcription. DAF-28 is the C. elegans insulin reported to be involved in regulation of the DAF-2/insulin receptor (Li et al., 2003). (B and C) An alternative model for transcriptional control of tph-1 is based on a similar one proposed by Seoane et al. (2004) for the control of cell proliferation in cultured mouse neuroepithelial and glioblastoma cells. In this model, the two isoforms of the DAF-16 transcription factor differentially regulate the activity of the DAF-3/SMAD protein. The DAF-16b isoform forms a complex with DAF-3 to activate transcription (B) whereas addition of the DAF-16a isoform results in either the release or the inactivation of the complex (C). The interaction between DAF-3 and DAF-16 may occur at a distance through apposition of their DNA binding sites via chromatin secondary structure although this is not directly represented.
the above findings, AKT-1 (Fig. 5A) and CREB, which are both positively regulated by CaM kinase phosphorylation (Soderling, 1999). The kinase AKT-1 acts downstream of the DAF-2/insulin receptor to inhibit DAF-16 (Nelson and Padgett, 2003) and is a known target of Cam kinases (Soderling, 1999), whereas the C. elegans gene encoding CREB, crh-1, is necessary for cyproheptadine-mediated upregulation of tph-1 (A. Estevez and M. Estevez, unpublished data). Because the epistatic analyses show that daf-16(rf) can suppress the ADF-W phenotype of both daf-4,unc-2daf-3 and unc-43(raf);unc-2 animals (Fig. 3E), it can be posited that DAF-16 may function as the ultimate downstream effector regulating the level of tph-1 expression through its inhibitory effects on the gene although other downstream effectors may also contribute to the overall regulation of tph-1 (such as DAF-3). Although the serotonergic signaling effector interacting with the UNC-43/CaMKII has not been identified, UNC-43/CaMKII may exert its effects through phosphorylation-mediated activation of either AKT-1, CRH-1, or both, and the partial suppression of the unc-43(gf);unc-2 ADF-S phenotype by daf-3 may represent the net effect of signaling from the UNC-2/UNC-43/CaMKII, DAF-7/TGF-β, and DAF-2/insulin cascades on DAF-16 (Fig. 5A).

The data presented here proposes that at least two growth factor-regulated pathways are involved in the stress-induced coordinate regulation of serotonin synthesis through antagonistic control of tryptophan hydroxylase transcription. Additionally, because the cyproheptadine-inhibited serotonin signaling can modulate expression of tph-1, TPH-1 activity itself ultimately integrates into this pathway to regulate its own gene through as yet undefined 5HT receptor(s) and their presumed effects on DAF-16 function. Pathophysiological disorders related to the synthesis and maintenance of serotonin include migraine (Estevez and Gardner, 2004), a severe form of headache that can be induced by environmental triggers such as hormonal fluctuations (Recober and Geweke, 2005), dietary headache that can be induced by environmental triggers such as disorders related to the synthesis and maintenance of serotonin presumed effects on DAF-16 function. Pathophysiological ultimately integrates into this pathway to regulate its own gene although other downstream effectors may also contribute to the overall regulation of tph-1 (such as DAF-3). Although the serotonergic signaling effector interacting with the UNC-43/CaMKII has not been identified, UNC-43/CaMKII may exert its effects through phosphorylation-mediated activation of either AKT-1, CRH-1, or both, and the partial suppression of the unc-43(gf);unc-2 ADF-S phenotype by daf-3 may represent the net effect of signaling from the UNC-2/UNC-43/CaMKII, DAF-7/TGF-β, and DAF-2/insulin cascades on DAF-16 (Fig. 5A).

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