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Calcineurin interacts with KIN-29, a Ser/Thr kinase, in *Caenorhabditis elegans*

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Abstract

Calcineurin is a Ca^{2+}/Calmodulin activated Ser/Thr phosphatase that is well conserved from yeast to human. In *Caenorhabditis elegans*, *tax-6* and *cnb-1* encode catalytic and regulatory subunits of calcineurin, respectively. We performed yeast two-hybrid screening using TAX-6 as a bait to identify calcineurin interacting proteins. KIN-29 is one of proteins that specifically interacted with TAX-6. KIN-29 is a Ser/Thr kinase previously shown to be involved in regulating gene expression of a subset of chemoreceptors in specific neurons. Both TAX-6 and KIN-29 are expressed in hypodermis, muscles, and neurons. Moreover, both calcineurin and *kin-29* mutants exhibit similar phenotypes, namely small body size, small brood size, and slow growth. Here we describe specific genetic interaction between *tax-6* and *kin-29* in regulating body size, serotonin mediated egg laying, and chemoreceptor expression.

Keywords: TAX-6; CNB-1; STR-2; Egg laying; Body size; MEF-2; Chemoreceptor expression

Calcineurin (Cn) is a Ca^{2+}/Calmodulin activated Serine/Threonine phosphatase which functions in diverse tissues such as T cells [1], neurons [2], and muscles [3]. Cn is composed of two subunits: catalytic subunit, calcineurin A (CnA), and regulatory subunit, calcineurin B (CnB). Overexpression of CnA causes cardiac hypertrophy [4] whereas knock-out of CnA leads to defective kidney development in mice [5] suggesting that proper regulation of Cn is important. *Caenorhabditis elegans* genome contains single CnA homolog, *tax-6*, and single CnB homolog, *cnb-1*.

Caenorhabditis elegans is an excellent model organism to study neurobiology, because genetic manipulation can be carried out easily and the function of individual neuron can be studied at single cell resolution. Egg laying is one of the behaviors that has been well characterized in *C. elegans* [6]. The egg laying apparatus in *C. elegans* is relatively simple and has been extensively studied. A pair of hermaphrodite specific neurons (HSN) innervates the vulval muscle and secretes the neurotransmitter, serotonin (5-HT), which stimulates the vulval muscle to contract, leading to the expulsion of eggs from the uterus. Environmental cues play important role in regulating egg laying process. For example, *C. elegans* stops laying eggs when food becomes scanty and resumes laying eggs when the food is available. We have previously reported that Cn positively regulates egg laying in *C. elegans* [7].

Cn regulates the activity of diverse proteins by dephosphorylation of phospho group attached at Ser/Thr residues [8,9]. MEF2, a MADS box transcription factor, is one of the proteins which is directly dephosphorylated and thereby activated by mammalian Cn [10]. MEF2 is a key transcription factor required for the development of muscle in fly and mammals [11,12]. However, in *C. elegans*, MEF-2 is not required for the development of muscle [13].

Abbreviations: lf, loss-of-function; gf, gain-of-function.

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Here we report for the first time that TAX-6 physically interacts with KIN-29, a Ser/Thr kinase that regulates body size, lifespan, dauer-entry, and expression of some of the chemoreceptors in the amphid neurons [14,22]. We extended our investigation into the genetic interactions between *tax-6*, *kin-29*, and *mef-2* with regard to some of the phenotypic abnormalities observed in *tax-6* loss-of-function mutants [*tax-6(lf)*] and/or *kin-29(lf)* mutants such as chemoreceptor expression, body length regulation, and serotonin mediated egg laying process.

Materials and methods

Genetics and maintenance of C. elegans. The following *C. elegans* strains were used in this work: N2, KM129 *mef-2(gv1lf)* I, KM130 *mef-2(gv2lf)* I, CX3695 *kyIs140[str-2::gfp]* I, CX3465 *kyIs39[sra-6::gfp]* I, IK17 *tax-6(p675lf)* IV, KJ306 *tax-6(jh107gf)* IV, PY3018 *pkIs41[kin-29::gfp]* IV, KJ300 *cnb-1(jh103null)* V, PY1479 *kin-29(oy38lf)* X, VC589 *kin-29(gk288lf)* X, CX3553 *kyIs104[str-1::gfp]* X, and PY1456 *kin-29(oy38lf) kyIs104[str-1::gfp]* X. Worms were handled according to the established methods [15].

Yeast two-hybrid analysis. Yeast two-hybrid screening was performed using full length of TAX-6 cloned into pAS2-1 as bait and *C. elegans* cDNA library cloned into pACT as prey vector. The screening was done as per the manufacturer's protocol (Clontech).

GST-pull down assay. GST-pull down assay was performed essentially as described previously [16]. Briefly, recombinant GST or GST fused TAX-6 was immobilized on Glutathione-Sepharose beads. One milligram of the extract obtained from mixed population of the strain expressing KIN-29 fused with GFP (PY3018 *pkIs41[kin-29::gfp]* IV) was mixed with either GST or GST fused TAX-6 which is immobilized on Glutathione-Sepharose bead. Unbound proteins were washed three times with binding buffer (50 mM Tris–HCl (pH7.4), 100 mM NaCl, 2 mM MgCl2, 0.2% Triton X-100, 0.5 mg/ml of bovine serum albumin, and 0.5 μM β-mercaptoethanol) and subjected to Western blot analysis using anti-GFP antibody.

Measurement of fluorescence intensity and body length. The intensity of the green fluorescence emitted from the fixed area of various transgenic worms along the dendrites of AWB or AWC or ASH neurons was calculated by using the software from Carl Zeiss (Axiovision). Body lengths of the wild type and mutant worms were measured as described previously [17]. Serotonin mediated egg laying behavior was analyzed as described previously [6].

Results

The catalytic subunit of C. elegans calcineurin, TAX-6, physically interacts with KIN-29

Full length of catalytic subunit of *C. elegans* calcineurin, TAX-6, was used as a bait to identify its interacting partners by yeast two-hybrid system. Sequencing the interacting prey vectors revealed that the C-terminal 497 amino acids of KIN-29 constituted one of the clones. In order to refine the region of TAX-6 that binds with KIN-29, we used serially deleted forms of TAX-6 as bait and tested with the prey, KIN-29. As shown in Fig. 1A and B, both full length of TAX-6 and truncated TAX-6 that lacks autoinhibitory domain could specifically interact with

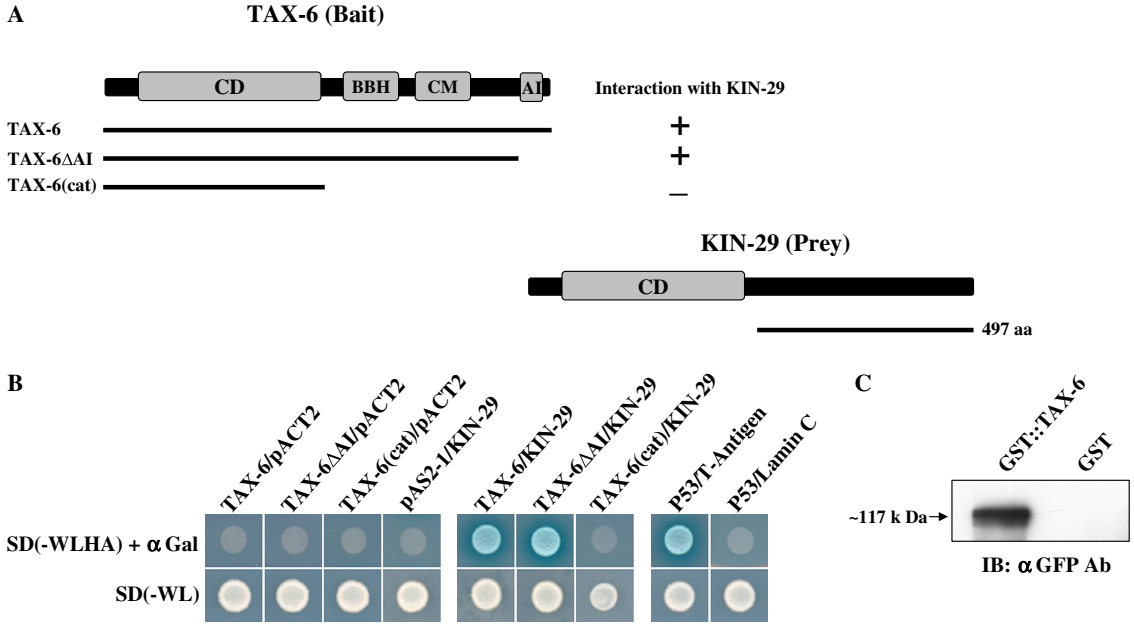

Fig. 1. (A) Schematic diagram of TAX-6 and KIN-29 showing the individual domains. Abbreviations used are: CD, catalytic domain; BBH, CnB binding Helix; CM, calmodulin binding motif; AI, auto inhibitory domain. The regions of the TAX-6 and KIN-29 covered by the bait vectors and prey vectors, respectively, are indicated as lines along with their ability (+) or inability (−) to bind with KIN-29 in yeast two-hybrid system. The diagram is not drawn to the scale. (B) Yeast two-hybrid analysis of the interaction between TAX-6 and KIN-29. The identity of the bait vectors and prey vectors co-transformed into the yeast is indicated as 'bait/prey' above each lane. Lower lanes indicate the ability of the co-transformants to grow on synthetic dropout media lacking Trp and Leu. Upper lanes indicate the ability (growth of blue colonies) or inability of the indicated transformants to activate three independent reporter genes. (C) GST::TAX-6 physically interacts with KIN-29::GFP. The soluble fraction of the extract obtained from the transgenic worms expressing KIN-29::GFP was applied on to either GST or GST::TAX-6 bound with Glutathione-Sepharose beads. After extensive washing to remove the unbound proteins, Western blot was performed utilizing anti-GFP antibody. A protein band which migrates at 117 kDa was detected only in the GST::TAX-6 lane.

KIN-29 as evidenced by the ability to form a blue colony on the synthetic dropout medium. Catalytic domain of TAX-6 did not activate the reporter gene when transformed with KIN-29, suggesting that KIN-29 does not bind with the catalytic domain of TAX-6. Previously, we have observed several other preys interact with catalytic domain of TAX-6 (unpublished results). Furthermore, the biochemically active catalytic domain of human CnA lacking CnB binding site ($CN_{\triangle 347}$) has been successfully purified by Chan et al. [24]. Given the high degree of amino acid identity between TAX-6 and human CnA (77%), we believe that the failure of the interaction between catalytic domain of TAX-6 and KIN-29 is not due to the improper expression and/or misfolding of the truncated form of TAX-6. These results indicate that part of C-terminal region of TAX-6 excluding catalytic domain specifically interacts with the C-terminal 497 amino acids of KIN-29.

In order to confirm the yeast two-hybrid results, we performed GST pull down assay. As shown in Fig. 1C, GST alone failed to pull down KIN-29::GFP whereas GST::TAX-6 specifically binds with KIN-29::GFP, supporting the data obtained from yeast two-hybrid system.

Calcineurin negatively regulates str-2 expression in AWC neuron

Expression of chemoreceptors such as STR-1, STR-2, and SRA-6 has been shown to be regulated by KIN-29 [14]. STR-1, STR-2, and SRA-6 are expressed in AWB, AWC and ASH neurons, respectively. Since AWC and ASH neurons express both KIN-29 and TAX-6 and since TAX-6 physically interacts with KIN-29, we asked whether calcineurin plays any role in modulating expression of the *str-2* and/or the *sra-6*. It is also known that wild type animal stochastically expresses STR-2 in only one of the two AWC neurons [18]. First, we have observed that there was no qualitative change in the *str-2* expression in any of the calcineurin mutants—all Cn mutants stochastically express the *str-2* in only one of the two AWC neurons (data not shown). In addition, we observed no gross defect in the morphology of the cilium or axons. These results indicate that Cn does not play any role in fate determination or differentiation of AWC neuron. However, in *tax-6(lf)* mutants and *cnb-1(null)* mutants, we found that the intensities of GFP expression along the dendrites were significantly brighter than wild type worms. On the other hand, *tax-6* gain-of-function mutant, *tax-6(gf)*, exhibited diminished level of *str-2* expression as visualized by GFP (Fig. 2A and B), suggesting that Cn negatively regulates *str-2* expression. Next, we examined expression of the *sra-6* in ASH neurons of Cn mutants. We found no qualitative or quantitative change in expression of the *sra-6* in any of Cn mutants (Fig. 2C). Also, we found no significant change in expression of the *str-1* in AWB neurons of Cn mutants. Taken together, Cn specifically and negatively regulates expression of the *str-2* in AWC neuron which is the opposite of KIN-29.

Genetic interaction between calcineurin, mef-2, and kin-29 in body size regulation

One of the most obvious phenotypic similarities between calcineurin loss-of-function mutants and *kin-29(lf)* mutants is small body size. As shown in Tables 1 and 3, *tax-6(lf)*, *cnb-1(null)*, and *kin-29(lf)* mutants are smaller than wild type worms, indicating that Cn and *kin-29* positively regulate body length in *C. elegans*. Interestingly, both double mutants, *tax-6(p675lf);kin-29(oy38lf)* and *tax-6(p675lf);kin-29(gk288lf)*, exhibit synergistically smaller body size, suggesting that *tax-6* genetically interacts with *kin-29* in regulating body size. Second, we tested the genetic interaction between *mef-2* and Cn mutants. The body length of *mef-2(gv2lf)* mutants is indistinguishable from that of wild type worms. However, the mutation in *mef-2* enhances the small body size of *tax-6(p675lf)* and *cnb-1(jh103null)* mutants, indicating the genetic interaction between *tax-6* and *mef-2*. Collectively, the observed synergism and enhancement arising from the combination of loss-of-function alleles suggest that *tax-6*, *kin-29*, and *mef-2* are acting on the same pathway in regulating body size. Finally, we examined the relationship between *kin-29* and *mef-2*. The small body size of *kin-29(gk288lf)* is completely suppressed by concomitant loss-of-function mutation in *mef-2*. This result is reminiscent of the *str-1* regulation by *mef-2*; the expression of *str-1* is reduced in *kin-29(lf)* mutants and this phenotype is suppressed by mutation in *mef-2* (P. Sengupta, personnel communication). Taken together, both the results indicate that KIN-29 inhibits MEF-2. So, in *kin-29(lf)* mutant, MEF-2 is not inhibited and the resultant higher activity of MEF-2 negatively regulates the body size.

Regulation of serotonin mediated egg laying by calcineurin, kin-29, and mef-2

Wild type worms lay eggs in response to exogenous supply of serotonin. However, as we have reported previously [19] and shown here in Table 4, *tax-6(lf)* and *cnb-1(null)* mutants lay smaller number of eggs in response to exogenous serotonin, In contrast, *tax-6(gf)* mutants lay larger number of eggs in response to serotonin, indicating that calcineurin positively regulates egg laying behavior in response to 5-HT. Since TAX-6 physically interacts with KIN-29, we asked whether *kin-29(lf)* mutants have any defect associated with egg laying in response to 5-HT. Utilizing two different alleles of *kin-29*, here we show for the first time that, indeed, *kin-29(lf)* mutants is resistant to serotonin mediated egg laying, suggesting that *kin-29* also positively regulates 5-HT-mediated egg laying. The epistatic analysis shows that the phenotype of *tax-6(gf);kin-29(lf)* double mutants are similar to that of *kin-29(lf)* mutant which suggests that *kin-29* is downstream of *tax-6* in executing 5-HT-mediated egg laying. Since MEF2 is a substrate of Cn in mammals, we examined the egg laying behavior of *mef-2(lf)* mutants. We report here for the first

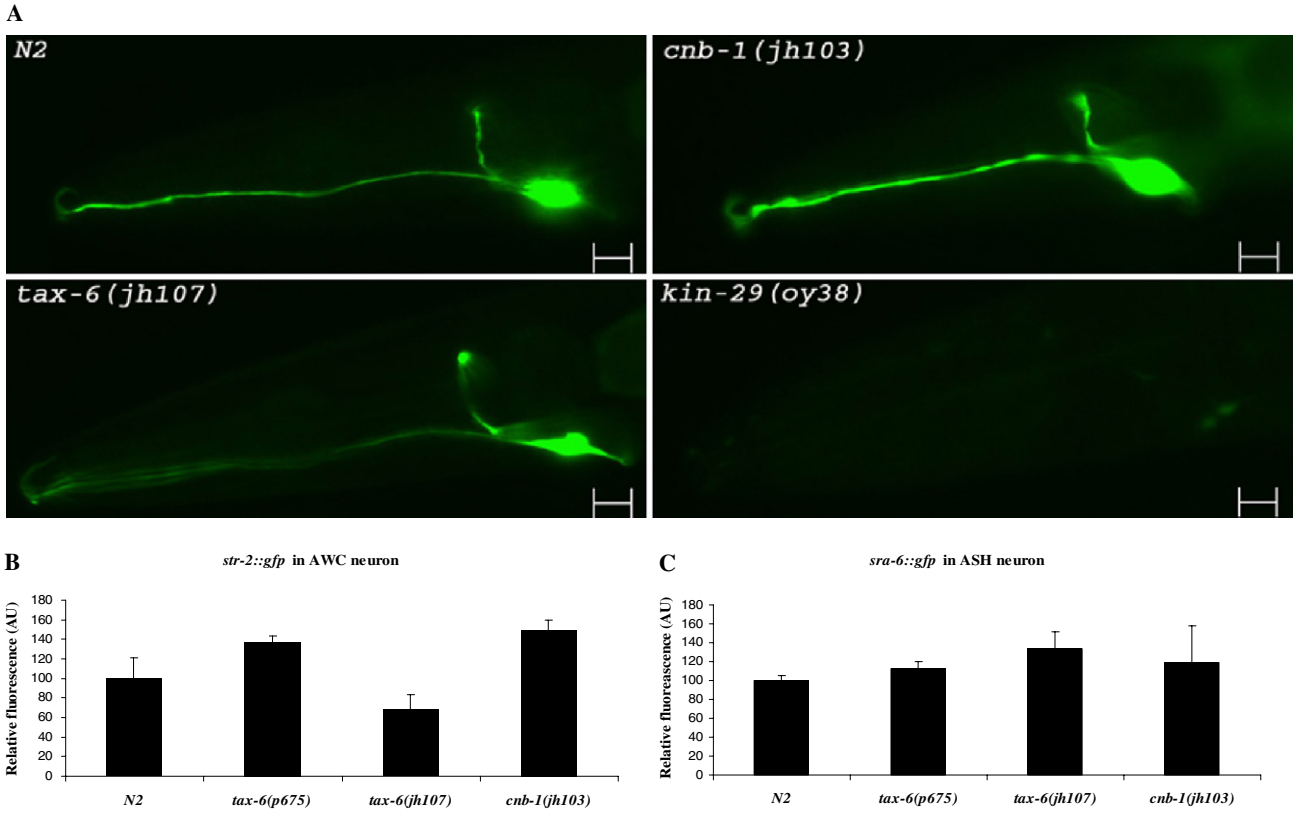

Fig. 2. (A) Calcineurin negatively regulates *str-2* expression. AWC neuron expressing GFP driven by *str-2* promoter under indicated genetic background. Anterior end of the worm is located in the left side of the picture. The intensity of fluorescence in *cnb-1(jh103null)* mutant is brighter than wild type, N2 worm. The *tax-6(jh107gf)* mutants show reduced level of *str-2* expression, especially in the dendrite. The scale indicates 10 μm. (B) Quantitative analysis of the fluorescence along the dendrites of AWC neurons expressing *str-2::gfp* under indicated genetic background. (C) Quantitative analysis of the fluorescence along the dendrites of ASH neurons expressing *sra-6::gfp* under indicated genetic background.

Table 1
Phenotypic similarities between *tax-6(lf)* mutants and *kin-29(lf)* mutants

tax-6(lf)	*kin-29(lf)*
Small body size [16,19,20]	Small body size [14,22]
Small brood size [16,19]	Small brood size [22]
Slow growth [20]	Slow growth [14,22]
Abnormal olfaction [20]	Defective sensory signaling [14]
Egg laying defective[a]	Egg laying defective[a]

[a] In this study.

time that *mef-2(gv1lf)* and *mef-2(gv2lf)* mutants are resistant to 5-HT. The direct conclusion from this result would be that *mef-2* positively regulates 5-HT-mediated egg laying. However, as explained below and in discussion, *mef-2* has a dual and opposing role in egg laying. The *kin-29(oy38lf)* and *kin-29(gk288lf)* mutants are highly resistant to 5-HT. Interestingly, the double mutants *mef-2(lf);kin-29(lf)* are moderately sensitive to 5-HT, indicating that mutation in *mef-2* suppresses *kin-29(lf)* phenotype. Regardless of the *mef-2* alleles or *kin-29* alleles utilized in our study, we found suppression of *kin-29(lf)* phenotype by *mef-2(lf)*. So, although the single mutants, *mef-2(lf)* and *kin-29(lf)*, are resistant to 5-HT, the double mutants between them are better than *kin-29(lf)* mutants in terms of their sensitivity towards 5-HT. This result suggests (i) the existence of MEF-2 mediated inhibitory pathway for controlling the 5-HT-mediated egg laying and (ii) KIN-29 negatively regulates MEF-2. Hence, in *kin-29(lf)* mutant, MEF-2 is not inhibited and the higher activity of MEF-2 participates in the inhibitory circuit controlling egg laying (Fig. 3). However, in *mef-2(lf);kin-29(lf)* double mutants, MEF-2 does not participate in this inhibitory circuit and hence the severity of 5-HT resistance is milder than *kin-29* single mutant. Next, we examined the genetic interaction between *mef-2* and *tax-6*. The *mef-2(gv1lf);tax-6(jh107gf)* and *mef-2(gv2lf);tax-6(jh107gf)* double mutants are hypersensitive to serotonin, which is similar to the phenotype of *tax-6(gf)* mutant, indicating that *tax-6* is downstream of *mef-2* in regulating egg laying process.

Discussion

We performed yeast two-hybrid screening to identify the proteins interacting with TAX-6 and we found that KIN-29 is one of the proteins that specifically interacts with TAX-6. GST-pull down assay confirmed the physical interaction between TAX-6 and KIN-29. Both TAX-6 and KIN-29 are expressed in neurons, muscles, and hypodermal seam cells [14,20]. Specifically some of the amphid

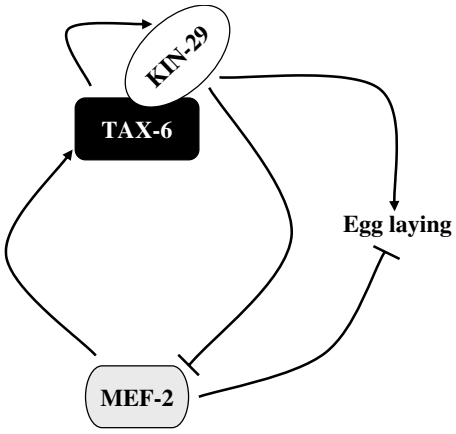

Fig. 3. Schematic model illustrating the regulation of serotonin mediated egg laying behavior. TAX-6 physically interacts with, and possibly activates, KIN-29. KIN-29 acts downstream of TAX-6 to execute egg laying process in response to 5-HT. MEF-2 plays a dual role in regulating egg laying process—on one hand MEF-2 acts upstream of TAX-6 and positively regulates TAX-6; on the other hand, MEF-2 inhibits egg laying in response to 5-HT and KIN-29 negatively regulates MEF-2.

neurons (see Table 2) express both TAX-6 and KIN-29. Expression of TAX-6 and KIN-29 in the same special confinement suggests that the observed physical interaction might be physiologically relevant.

In addition to the physical interaction, we presented several evidences for genetic interaction between calcineurin and *kin-29*. First, several phenotypes of *tax-6* loss-of-function mutants and *kin-29* loss-of-function mutants are similar (see Table 1) which indicates that both *tax-6* and *kin-29* are regulating similar pathway. Second, the *cnb-1(jh103null);kin-29(oy38lf)* double mutants is synthetic lethal which suggests that the viability of *C. elegans* is dependent on at least one of the two genes. Third, the body length of *tax-6(p675lf);kin-29(oy38lf)* double mutants are synergistically smaller than that of individual mutant. Fourth, *kin-29* is epistatic to *tax-6* in 5-HT-mediated egg laying.

What is the consequence of the physical interaction between TAX-6 and KIN-29? Our genetic data suggest that KIN-29 acts downstream of TAX-6 in regulating 5-HT-mediated egg laying, raising the possibility that TAX-6 activates KIN-29.

KIN-29 positively regulates the transcription of *str-1::gfp* in AWB neuron. As reported by A. Lanjuin and P. Sengupta [14], and shown here in Fig. 2A, the *kin-29(oy38lf)* mutants show dramatic reduction in *str-2* expression. In contrast, the observed changes in *str-2* expression in calcineurin mutants are rather subtle and obvious only along the dendrites. It seems that perhaps, Ca^{2+} signaling, in general, may negatively regulate *str-2* expression in AWC neuron. Ca^{2+} signaling components such as the Ca^{2+} channel proteins (UNC-2, UNC-36), Ca^{2+}/calmodulin-dependent protein kinase (UNC-43) have been shown to inhibit the onset of *str-2* expression in AWC neuron [18]. Also, we have previously reported that *tax-6* genetically interacts with *unc-43* [21]. Thus the observed inhibition of *str-2* expression by Cn could be due to the general perturbation of Ca^{2+} signaling.

The 5-HT-mediated egg laying is a complex process as depicted in Fig. 3. TAX-6 physically interacts with and possibly activates KIN-29. MEF-2 exerts dual and opposing role in regulating egg laying behavior; through the positive regulation of TAX-6, it stimulates egg laying process, thus in the *mef-2* mutants, diminished level and/or activity of TAX-6 could account for the resistance to serotonin. In

Table 3
Body size of indicated mutants

Genotype	Average body length (μm)	%	*n*
N2(WT)	1269 ± 70.2	100	50
tax-6(p675lf)	1047 ± 79.5	82.5	27
cnb-1(jh103null)	901.9 ± 72.0	71	50
kin-29(oy38lf)	1201.1 ± 101.6	94.6	36
kin-29(gk288lf)	1016.7 ± 80.5	80.1	35
tax-6(p675lf);kin-29(oy38lf)	787.6 ± 69.2	62.1	37
tax-6(p675lf);kin-29(gk288lf)	753.1 ± 62.6	59.3	31
mef-2(gv2lf)	1306.1 ± 66.7	102.9	50
mef-2(gv2lf);tax-6(p675lf)	896.9 ± 61.5	70.7	50
mef-2(gv2lf);cnb-1(jh103null)	754.6 ± 83.3	59.5	50
mef-2(gv1lf)	1282.3 ± 65.2	101	46
mef-2(gv1lf);kin-29(gk288lf)	1288.8 ± 55.2	101.6	50

Table 2
Summary of neurons expressing both TAX-6 and KIN-29, and their major functions [23]

Neurons	Receptors expressed	Functions
AFD	OSM-9, OCR-2, SRB-6, SRD-1	Thermotaxis
AWA	ODR-10, OSM-9, OCR-1, OCR-2	Chemotaxis to diacetyl, pyrazine, 2,4,5-trimethylthiazole
AWC	STR-2, OSM-9, DAF-11	Chemotaxis to benzaldehyde, butanone, isoamyl alcohol, 2,3-pentanedione, 2,4,5-trimethylthiazole
ASH	SRA-6, OSM-9, OCR-2, SRB-6, NPR-1, UNC-8	Avoidance to nose touch, hyperosmolarity, and to 1-octanol
ASK	OSM-9, SRA-7, SRA-9, SRG-2, SRG-8, DAF-11	Chemotaxis to lysine
ASI	SRD-1, STR-2, STR-3, DAF-11	Chemotaxis to lysine; controls entry into dauer stage
AIY	SER-2, SRA-11	Thermotaxis
AIZ	SER-2	Thermotaxis

Table 4
Characterization of serotonin mediated egg laying phenotype

Genotype	No. of animals laying the indicated number of eggs after treatment with							
	M9 buffer				Serotonin			
	0	1–3	4–7	>7	0	1–3	4–7	>7
N2(WT)	48	0	0	0	2	17	10	9
tax-6(p675lf)	48	0	0	0	20	13	15	0
cnb-1(jh103null)	46	2	0	0	37	11	0	0
tax-6(jh107gf)	48	0	0	0	3	3	13	29
kin-29(oy38lf)	48	0	0	0	46	1	1	0
kin-29(gk288lf)	48	0	0	0	36	11	1	0
tax-6(jh107gf);kin-29(oy38lf)	48	0	0	0	17	26	5	0
mef-2(gv1lf)	47	1	0	0	31	14	1	2
mef-2(gv2lf)	48	0	0	0	19	20	7	2
mef-2(gv1lf);tax-6(jh107gf)	47	1	0	0	2	0	5	41
mef-2(gv2lf);tax-6(jh107gf)	44	4	0	0	0	1	6	41
mef-2(gv1lf);kin-29(oy38lf)	48	0	0	0	34	10	4	0
mef-2(gv1lf);kin-29(gk288lf)	47	1	0	0	4	29	8	7
mef-2(gv2lf);kin-29(oy38lf)	48	0	0	0	32	9	7	0
mef-2(gv2lf);kin-29(gk288lf)	48	0	0	0	34	10	3	1

kin-29 mutants, direct activation of egg laying process by KIN-29 is abolished. Additionally MEF-2 is no longer inhibited by KIN-29. Higher activity of MEF-2 inhibits egg laying through a separate pathway. Although the higher MEF-2 activity should in principle provide a strong signal to lay more eggs by activating TAX-6, the absence of the final executer of egg laying, KIN-29, does not complete the egg laying circuit, rendering them resistant to 5-HT.

Acknowledgments

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