学位論文

Non-redundant function of two subtypes of octopamine receptors in food deprivation-mediated signaling in *C. elegans*

(線虫の餌非存在下で活性化されるシグナルにおける
2種類のオクトパミン受容体の非重複的な機能)

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申請

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Abbreviations

3'UTR	3' untranslated region
Bp	base pairs
cAMP	cyclic adenosine monophosphate
C. elegans	*Caenorhabditis elegans*
cDNA	complementary deoxyribonucleic acid
CREB	cAMP response element-binding protein
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GFP	green fluorescent protein
HA	hemagglutinin
HEK cell	human embryonic kidney cell
mRNA	messenger ribonucleic acid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RT	reverse transcription
TBS	Tris-buffered saline
TM	transmembrane domain
Tris	tris(hydroxymethyl)aminomethane

A	adenosine
C	cytidine
G	guanosine
T	thymidine

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Abstract

Amine neurotransmitters act primarily through G protein-coupled receptors (GPCRs). In many cases, there are multiple receptors that bind to the same neurotransmitter and activate the same intracellular signaling cascades. In a model animal *Caenorhabditis elegans*, four amine neurotransmitters, octopamine, tyramine, dopamine and serotonin function in neurons and muscles to modulate behaviors and metabolism in response to environmental cues. It has been previously shown that octopamine activates CREB (cAMP response element-binding protein) in the cholinergic SIA neurons during food deprivation through activation of the octopamine receptor SER-3 (serotonin/ octopamine receptor family member 3) in these neurons. In the present thesis, I analyzed another subtype of octopamine receptor, SER-6 (serotonin/ octopamine receptor family member 6), which is highly homologous to SER-3. As seen in *ser-3* deletion mutants, CREB activation induced by exogenous octopamine and food deprivation was decreased in *ser-6* deletion mutants compared to wild-type animals, suggesting that SER-6 is required for signal transduction. Expression of SER-6 in the SIA neurons was sufficient to restore CREB activation in the *ser-6* mutants, indicating that SER-6 functions in these neurons as does SER-3. Furthermore, the response to exogenous octopamine and food deprivation was not different between *ser-3/+;ser-6/+* double heterozygous animals and wild-type animals. *ser-3* mutants overexpressing SER-6 responded to exogenous octopamine and food deprivation as weakly as *ser-3* mutants. Moreover, overexpressed SER-3 in *ser-6* mutants did not fully restore CREB activation. Taken together, these results demonstrate that two types of similar GPCRs, SER-3 and SER-6, are required for normal signaling and function in the same cells in a non-redundant manner.

Contents

Introduction

G protein-coupled receptors (GPCRs) constitute a large family of seven transmembrane proteins. They detect and respond to extracellular signals such as neurotransmitters, hormones, odorants and photon. Binding of ligand to the GPCR activates a heterotrimeric G protein that consists of three subunits, α, β, and γ. Gα subunit is a GTPase switch that alternates between the GTP-bound active state and the GDP-bound inactive state. The active form of Gα subunit dissociates from the $G_{\beta\gamma}$ subunits, and both of them are considered to activate various effectors.

G protein activates different intracellular signal pathways corresponding to Gα subtypes. Gαs-coupled receptor activates adenylyl cyclase, which synthesizes the second messenger cAMP. The effect of cAMP is mediated through activation of protein kinase A (PKA). The activated PKA subunit translocates to the cell nucleus, where it phosphorylates and activates cAMP response element-binding protein (CREB), which is a transcription factor. By contrast, Gαi-coupled receptor inhibits adenylyl cyclase. Gαq-coupled receptors activate phospholipase C (PLC) and PLC then hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol trisphosphate (IP3). DAG activates Protein Kinase C (PKC). IP3 binds to an IP3 receptor, a calcium channel, causing release of Ca^{2+} from endoplasmic reticulum (ER). In general, CREB activation is dependent on Gs through PKA signaling pathway. However, some studies suggest that Gq can also activate CREB. For example, it has been reported that activation of α adrenergic-like receptors such as octopamine receptors of insects leads to the elevation of both Ca^{2+} and cAMP in response to octopamine in a heterologous expression system, although it is unclear whether the Gq–CREB pathway is of physiological relevance (Lin et al., 1998; Chalecka-Franaszek et

al., 1999; Thonberg et al., 2002; Huang et al., 2010; Han et al., 1998; Grohmann et al., 2003). One possibility is that Ca^{2+} released from ER in response to Gq signaling may activates adenylyl cyclases which are stimulated by Ca^{2+}/calmodulin or activates Ca^{2+}/calmodulin-dependent protein kinase, resulting in activation of CREB.

GPCRs also activate intracellular signals through the $G_{\beta\gamma}$ subunit. Ligand binding leads to dissociation of $G_{\beta\gamma}$ subunit from $G\alpha$ and released $G_{\beta\gamma}$ subunit then binds to and opens K^+ channels. The increase in K^+ permeability hyperpolarizes the cellular membrane.

Amine neurotransmitters, such as dopamine, noradrenaline, and serotonin, signal primarily through GPCRs. Each neurotransmitter is capable of binding to multiple types of receptors, which in turn couple to different G proteins, allowing a single neurotransmitter to activate multiple intracellular signaling pathways. It is common for multiple receptors to allow binding of the same neurotransmitter and activate the same intracellular signaling cascades. The α_1-adrenergic receptors, for example, consist of three subtypes, α_{1a}, $\alpha_{1b,}$ and α_{1d}. All three receptors allow binding of both adrenaline and noradrenaline, and couple to G protein Gq.

Recent studies have shown that different subtypes of GPCRs are capable of regulating each other through the formation of heterodimers *in vivo*, and in doing so acquire new functions (Gupta et al., 2010; He et al., 2011; Pei et al., 2010). Functionally similar receptors have been shown to form heterodimers when expressed heterologously in cultured cells, suggesting that these types of receptors can work cooperatively. For example, the α_{1b}-adrenergic receptor facilitates internalization of the α_{1a}-adrenergic receptor by forming a hetero-oligomer without affecting the pharmacology or signaling of each receptor (Stanasila et al., 2003). Similarly, the α_{1b}-adrenergic receptor is capable

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of forming a heterodimer with the α_{1d}-adrenergic receptor, facilitating its expression on the cell surface (Hague et al., 2004). This heterodimer behaves as a single functional entity with increased level of signaling (Hague et al., 2006). The α_{2a}-adrenergic receptor and the α_{2c}-adrenergic receptor form a heterodimer reducing GRK-mediated receptor phosphorylation and β-arrestin recruitment, affecting downstream Akt phosphorylation (Small et al., 2006). Moreover, the β_1-adrenergic receptor and the β_2-adrenergic receptor form a heterodimer, regulating sensitization to agonist stimulation and suppression of the spontaneous activity of β_2-adrenergic receptor in cardiac myocytes, optimizing β-adrenergic modulation of cardiac contractility (Zhu et al., 2005). Together, these interactions suggest that similar receptors may perform non-redundant functions when expressed in the same cell.

C. elegans is a small, free-living nematode that lives in temperate soil environments. It primarily feeds on bacteria and reproduces within about 3 days at 20°C (Wood, 1988). *C. elegans* has been used as a model organism to investigate gene functions *in vivo*, since deciphering its genome sequence is essentially complete (*C. elegans* Sequencing Consortium, 1998), introducing foreign DNA into worms is relatively easy, and various genetic tools are available. The transparency of *C. elegans* allows for visualization of GFP-tagged proteins by fluorescent microscopy in living animals. Furthermore, it is a very simple organism; the adult hermaphrodite has only 302 neurons, and the structure and connectivity of the nervous system of *C. elegans* has been documented from reconstructions of electron micrographs of serial sections (White et al., 1986). Therefore, it is a suitable model animal for the *in vivo* analysis of the neural functions.

The nomenclature of neurons is fairly arbitrary; neuron's names are used to identify the neuron class. The 302 neurons are classified into 118 categories based on their morphology and synaptic connectivity. Each neuron's name consists of two or three upper case letters (e.g. SIA). The suffix letters L, R, D, and V represent left, right, dorsal, and ventral, respectively. For example, neuron class SIA has neurons SIADL, SIADR, SIAVL and SIAVR (Wood, 1988).

Four biogenic amines of *C. elegans,* octopamine, tyramine, dopamine and serotonin act to modulate behaviors and metabolism in response to environmental cues (Chase and Koelle, 2007). Octopamine is considered to be the biological equivalent of mammalian noradrenaline (Roeder, 1999). It is synthesized by tyramine β-hydroxylase, TBH-1, and antibody staining of TBH-1 revealed that octopamine is synthesized only in the RIC interneurons and the gonadal sheath cells (Alkema et al., 2005). In *C. elegans*, exogenous octopamine inhibits egg laying and pharyngeal pumping (Horvitz et al., 1982; Alkema et al., 2005; Chase and Koelle, 2007), and the application of octopamine to well-fed animals suppresses thermotaxis to a memorized temperature (Mohri et al., 2005). Thus, responses mediated by exogenous octopamine and food deprivation are similar, suggesting that octopamine signaling opposes the signal induced by food (Sasakura et al., 2013). In fact, octopamine activates CREB in food-deprived *C. elegans*, and this activation was suppressed by dopamine signaling that is activated by bacterial food (Suo et al., 2006; Suo et al., 2009). Octopamine also modulates metabolism in *C. elegans*; octopamine is required for serotonin-mediated fat decrease, and octopamine and serotonin work synergistically in this regulation (Srinivasan et al., 2008; Noble et al., 2013).

The *C. elegans* genome contains at least three octopamine receptors, SER-3, SER-6 and OCTR-1. SER-3 is a putative Gq-coupled octopamine receptor, since it increases intracellular Ca^{2+} concentration in response to 10 nM octopamine when expressed in HEK 293 cells (Petrascheck et al., 2007). SER-3 mediates lifespan-extending effects of mianserin, an antidepressant, on *C. elegans* (Petrascheck et al., 2007), which is believed to be related to dietary restriction signaling. *ser-3* is required for inhibition of movement by serotonin, with *ser-3* mutants being hyperactive. An RNAi study also suggested the involvement of *ser-3* in regulation of brood sizes, embryonic development, and pharyngeal pumping (Carre-Pierrat et al., 2006).

SER-6 has been shown to accept binding of octopamine, and is believed to be coupled to Gq, due to its ability to activate inward currents upon octopamine treatment when heterologously expressed in *Xenopus* oocytes, which presumably is mediated by opening of endogenous Ca^{2+}-gated chloride channels (Mills et al., 2012). SER-6 functions in AWB chemosensory neurons to control serotonin-induced fat decrease by stimulating serotonin production, which induces transcriptional regulation of lipid oxidation genes (Srinivasan et al., 2008; Noble et al., 2013). SER-6 inhibits aversive responses against 30% 1-octanol by stimulating the release of neuropeptides that activate receptors on sensory neurons mediating attraction or repulsion (Mills et al., 2012).

OCTR-1 was identified as a Gi-coupled octopamine receptor, using a heterologous expression in *Xenopus* oocytes (Mills et al., 2012). The tyramine receptor TYRA-3 has also been shown to allow binding of octopamine, albeit weakly.

Previously, Suo et al. showed that amine neurotransmitters regulate activation of

CREB in *C. elegans* (Suo et al., 2006; Suo et al., 2009). CREB is a transcription factor that plays essential roles in a variety of biological processes (Lonze and Ginty, 2002; Johannessen et al., 2004). It binds to specific DNA sequences called cAMP response elements (CRE) and regulates expression of its target genes upon phosphorylation (Mayr and Montminy, 2001). In *C. elegans*, CREB activation can be detected by fluorescence in animals carrying a *cre::gfp* reporter, in which CRE is fused to a GFP sequence (Kimura et al., 2002). Using this reporter for CREB activation, Suo et al. previously found that CREB is activated in the cholinergic SIA neurons in the absence of food (Suo et al., 2006). This signaling is mediated by the amine neurotransmitter octopamine since food deprivation-mediated CREB activation was decreased in octopamine-deficient mutant *tbh-1* and CREB can be activated by the addition of exogenous octopamine. SER-3, a putative Gq-coupled octopamine receptor, and EGL-30, an α subunit of Gq, function in the SIA neurons to induce CREB activation (Fig.1). Furthermore, this octopamine signaling is suppressed by dopamine, since exogenous dopamine suppressed CREB activation in the presence of exogenous octopamine and CREB was spontaneously activated in the presence of food in dopamine-deficient mutant *cat-2*. *cat-2* encodes a tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine. Dopaminergic neurons of *C. elegans* possess sensory endings that are exposed to external milieu, which senses mechanical stimulus of bacterial food and this sensation causes dopamine release. Dopamine functions through activation of the dopamine receptors DOP-2 and DOP-3 (Suo et al., 2009). Moreover, the regulation of CREB activation by amine neurotransmitters in SIA neurons modulates acetylcholine release from these neurons (Suo and Ishiura, 2013).

SER-6 was presumed to be highly homologous to SER-3. SER-6 has been shown to bind octopamine and is believed to be coupled to Gq (Mills et al., 2012) as is SER-3. In the present thesis, I cloned *ser-6* and analyzed the involvement of SER-6 in octopamine-mediated CREB activation and found that SER-6 functions in SIA neurons, similar to SER-3. Interestingly, loss of either SER-3 or SER-6 leads to diminished signaling, indicating that both receptors are required for normal signaling. These two similar octopamine receptors are therefore working in the same cells, and function in a non-redundant manner *in vivo*.

Texts on p.7, l.9 - p.8, l.11 and p.10, l.24 - p.12, l.8 are the pre-peer reviewed version of the following article: Yoshida M, Oami E, Wang M, Ishiura S, Suo S. 2014. Nonredundant function of two highly homologous octopamine receptors in food-deprivation-mediated signaling in *Caenorhabditis elegans*. J Neurosci Res., which has been published in final form at

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Materials and Methods

Culturing *C. elegans*

C. elegans was cultured on NGM agar plates containing 1.7% agar, 0.25% bacto-peptone (Becton Dickinson), 50mM NaCl, 0.1% cholesterol, 1mM $MgSO_4$, 1 mM $CaCl_2$ and 25 mM potassium phosphate buffer (pH 6.0), covered with *Escherichia coli* OP50 (Brenner, 1974) as a food at 20^oC. Animals were transferred to new plates once every 3-4 days before they exhaust the food and starve.

Mating of *C. elegans*

To obtain males, 15 young adult hermaphrodites were transferred to a NGM plate (10cm in diameter) and incubated at 30^oC for about 5 hours. For mating, 6-8 adult males and 2 young adult hermaphrodites were transferred to a NGM plate (3cm in diameter). Hermaphrodites were mated with males to obtain double or triple mutants, and to backcross worms with wild-type N2 strain.

Genotyping of *C. elegans*

Single worms were put in PCR tubes containing 40ng/μL Proteinase K, 50 μM KCl, 10 μM Tris (pH 8.2), 2.5 μM $MgCl_2$, 0.45% (v/v) NP-40, 0.45% (v/v) Tween 20 and 0.01% (w/v) Gelatin. They were lysed by incubating at 60^oC for 45 minutes and then Proteinase K was inactivated by additional incubation at 95^oC for 15 minutes. PCR was performed using target gene-specific primers which match upstream and downstream of their deleted regions (Table 1) with *Ex Taq* (TaKaRa) using diluted lysates as the template. The reaction solution contained 1 μL 10x *Ex Taq* buffer (TaKaRa), 0.2 mM

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dNTP, 1 μL diluted lysates, 0.5 μM forward primer, 0.5 μM reverse primer and 0.05 μL *Ex Taq* in 10μL. PCR condition was as follows; annealing temperature was 59°C, extension temperature was 72°C, extension times were generally 1 minute per kb and these reactions were repeated 30 times. The resulting PCR products were electrophoresed to determine the genotype of the animals. To genotype *cat-2(e1112) II*, PCR products were digested with *DdeI*, which only digests DNA from the *e1112* allele.

Strains

The alleles used in this study were as follows: *ser-3(ad1774) I* (Suo et al., 2006), *ser-6(tm2104) IV, ser-6(tm2146) IV* (gifts from the National BioResource Project [NBRP], Ministry of Education, Culture, Sports, Science and Technology [MEXT], Tokyo, Japan), *octr-1(ok371)X* (Wragg et al., 2007), *tyra-3(ok325) X* (Wragg et al., 2007), *unc-64(e246) III* (Brenner, 1974), *tbh-1(ok1196) X* (Suo et al., 2006), *cat-2(e1112) II* (Sulston et al., 1975) and *tzIs3*[*cre::gfp; lin-15(+)*] (Kimura et al., 2002). All the mutations were in the background of the wild-type Bristol N2 strain (Brenner, 1974). *octr-1* mutants and *tyra-3* mutants were backcrossed three times with N2. All mutants used in the CREB activity assay carried *cre::gfp* reporter. These mutants were generated by mating *tzIs3* males with other mutants. The resulting genotypes were confirmed by PCR. *tbh-1(ok1196);tzIs3*, *ser-3(ad1774);tzIs3*, and *unc-64(e246)III;tzIs3* were constructed previously (Suo et al., 2006).

Cloning of *ser-6*

The wild-type Bristol N2 were cultured at 20°C on 10 NGM plates (10cm in diameter), and a population of *C. elegans* at mixed stages were collected in M9 buffer

(0.2% Na_2HPO_4, KH_2PO_4, 0.5% NaCl, 1mM $MgSO_4$, pH7.0). Worms were washed once with M9 buffer. Five hundred microliter of TriZOL reagent (Gibco BRL) was added to 1 mL worm suspension and homogenized in liquid nitrogen using a mortar. Two milliliter of TriZOL reagent were added to the worm homogenate and mixed vigorously, and then incubated for 5 minutes at room temperature. After incubation, the sample was centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to a new 1.5 mL tube and mixed with 1/5 volume of chloroform. After centrifugation at 13,000 rpm at 4^oC for 15 minutes, the upper phase was transferred to an RNase-free tube and mixed with isopropanol. Ten minutes later, the sample was centrifuged at 13,000 rpm at 4^oC for 10 minutes. The supernatant was discarded and the pellet was washed with 75% ethanol and dissolved in RNase-free water.

The cDNA of SER-6 was synthesized using a gene-specific primer and the Prime Script 1st strand cDNA synthesis kit (TaKaRa). PCR was carried out using a SER-6 reverse primer and an SL1 primer matching the 5'-trans-spliced leader sequence found on *C. elegans* RNAs (Blaxter and Liu, 1996) using PfuUltra High-Fidelity DNA Polymerase (Stratagene). The sequences of primers used for cloning of *ser-6* are shown in Table 1. The resulting PCR product was cloned into pCR-Blunt (Invitrogen) and sequenced.

Phylogenetic analysis

The amino acid sequences of SER-6 and other biogenic amine receptors of human and invertebrates were aligned with ClustalW (DNA Databank of Japan), using relatively well-conserved regions excluding the N terminus, second extracellular loop, third intracellular loop, and the C terminus of these receptors. The phylogenic tree was

drawn with PHYLIP by the Fitch-Margoliash method and visualized with TreeView. Receptor sequences used and the GenBank accession numbers are as follows: *C. elegans* octopamine receptors (ceSER-3, NP491954 and ceOCTR-1, CCD83472.1), *C. elegans* dopamine receptors (ceDOP-1, CCD68411.1; ceDOP-2, CBY85347.1; ceDOP-3, NP_001024907.2 and ceDOP-4, CCD65696.1), *C. elegans* tyramine receptors (ceTYRA-2, CCD83463.1; ceTYRA-3, CCD83479.1 and ceSER-2, NP_001024335.1), *C. elegans* serotonin receptors (ceSER-1, CCD63419.1; ceSER-4, CCD73768.1 and ceSER-7, CCD83456.1), insect α-adrenergic-like octopamine receptors (dmOAMB, AAC17442; amOAMB, CAD67999; paOA1, AAP93817.1 and bmOAR1, NP_001091748.1), human dopamine receptors (hD1, P21728; hD2, P14416; hD3, P35462; hD4, P21917; and hD5, P21918), human serotonin receptors (h5HT1a, I38209; h5HT1b, JN0268; h5HT1d, A53279; h5HT1e, A45260; h5HT1f, A47321; h5HT2a, A43956; h5HT2b, S43687; h5HT2c, JS0616; h5HT4, Q13639; and h5HT7, A48881), and human adrenergic receptors (hα1A, NP000671; hα1B, NP000670; hα1D, NP000669; hα2A, A34169; hα2B, A37223; hα2C, A31237; hβ1, QRHUB1; hβ2, QRHUB2; and hβ3, QRHUB3). A human trace amine receptor 3 (hTAR3, AAO24660) was used as an out group.

Analyses of CRE-mediated gene expression

CREB activation assays were performed as described previously (Suo et al., 2006; Suo et al., 2009). Animals carrying *cre::gfp* were synchronized by a hypochlorite treatment, and the resulting eggs were placed on NGM plates seeded with *E. coli* OP50. Animals were incubated for 2 days at 20ºC, transferred to new NGM plates, and incubated for an additional 24 hours. Animals were then transferred onto assay plates,

and incubated for 4 hours at 20ºC. Each assay plate contained 1.7% AgarNoble (BD Diagnostics) with or without 3 mg/mL octopamine-hydrochloride (Sigma-Aldrich) with bacterial food spread on its surface. For food depletion assays, synchronized animals were incubated on NGM plates seeded with or without OP50 at 20ºC for 6 hours. For soaking assays, synchronized animals were incubated for 4 hours at 20ºC on 60 mm NGM plates seeded with bacterial food and overlaid with ~5 mL of water. After incubation, animals were collected in M9 buffer (Brenner, 1974) containing 50mM NaN$_3$ that paralyzes worms. Approximately 400 µL of molten 5% agarose in M9 buffer was put on a glass slide and another glass slide was placed on top of the agarose to make a thin layer. Slides were detached and paralyzed worms were put on the agar in a drop of the buffer. A cover slip was placed over the agarose layer and excess buffer was removed with a piece of filter paper. Slides were covered with saran wrap to prevent drying. The number of SIA neurons expressing GFP was counted for each animal using a fluorescence microscope (Olympus BX53) to quantify CREB activation. All counting was performed by an experimenter-blinded to the genotype and incubation conditions of the animals. Statistical significance was evaluated by an analysis of variance followed by a Tukey-Kramer multiple comparison test using GraphPad Prism (GraphPad Software). Images of animals were obtained using the fluorescence microscope.

Microinjection

Microinjection was performed as described (Mello et al., 1991) to create transgenic animals. Plasmid DNA mixtures (0.1 µg/µL) were filtered with Centrifugal filters (Durapore-PVDF 0.22µm, Millipore). To make injection needles, glass capillary GDC-1 (Narishige) was pulled by a needle puller (Narishige). About 1 µl of DNA injection mix

was loaded into an injection needle. Worms were transferred to a bacteria-free NGM plate to remove bacteria. Injection pad is a thin glass slide covered with a dried agarose layer, which was used to fix worms while they are injected. Worms stick to the pads due to the absorption of water from the body into the dry agarose. A drop of oil was placed on an injection pad and a worm was placed in the oil drop. The needle was inserted into gonads of hermaphrodites and the DNA mixtures were injected by pressure supplied by Microinjector FemtoJet (Eppendorf).

Isolation of genomic DNA

The wild-type Bristol N2 animals were cultured at 20°C on three NGM plates (10cm in diameter), and a population of *C. elegans* at mixed stages were collected with M9 buffer. Worms were washed with M9 buffer once and were frozen at -80°C and thawed. Lysis buffer (100mM NaCl, 100mM Tris HCl pH8.0, 50mM EDTA pH8.0, 1% SDS, 1% 2-Mercaptoethanol, 100μg/mL Proteinase K) was added to the worm suspension and incubated at 65°C for 1 hour. The lysate was purified by Phenol–chloroform extraction to obtain worm genomic DNA.

Analyses of *ser-6* expression patterns

The transcriptional reporter fusion gene *ser-6::gfp* was generated using the fusion PCR method as described (Hobert, 2002) using the primers Y54fusionA, Y54fusionB, Y54fusionE, fusionD, fusionF and fusionC (Fig.5). The primer sequences are shown in Table 1. The region corresponding to 5.0-kb upstream and a part of exon 1 of *ser-6* gene were amplified with the primers Y54fusionA and Y54fusionB with *LA Taq* (TaKaRa) using genomic DNA as the template. The region corresponding to *gfp* gene and 3'-UTR

of *unc-54* (2-1876) were amplified with the primers fusionC and fusionD with *LA Taq* using the plasmid pPD95.75 as the template. Using these PCR products and primers Y54fusionE and fusionF, 2nd PCR was performed and *ser-6* promoter fused to *gfp* gene was obtained (*ser-6::gfp*).

ser-6::gfp was injected into N2 wild-type animals together with *ceh-17::dsred* (Pujol et al., 2000; Suo et al., 2006), *tbh-1::dsred* (Alkema et al., 2005; Suo et al., 2006), pBluescript (Stratagene) and the transformation marker pRF4, which contains the dominant roller mutation *rol-6(su1006)* (Kramer et al., 1990). Concentrations of the injected plasmids were 30, 10, 10, 30, and 20 ng/µL, respectively. Images of transformants were obtained using a confocal laser microscope (Leica DMI6000 B).

Cell-specific rescue of *ser-6*

To express *ser-6* in the SIA neurons, cDNA of *ser-6* was fused to the *ceh-17* promoter, which induces gene expression in only the SIA and ALA neurons. The coding sequence of *ser-6* was amplified with the corresponding forward and reverse primers with PfuUltra High-Fidelity DNA Polymerase (Stratagene) using subcloned *ser-6* cDNA as the template. The sequences of primers used for construction are shown in Table 1. The PCR product was digested with the restriction enzymes *Age*I and *Not*I and cloned into *Age*I- and *Not*I-digested *ceh-17::dop-2l* (Suo et al., 2009) to obtain *ceh-17::ser-6*. *ceh-17::ser-6* was then injected into *ser-6(tm2104);tzIs3* together with transformation marker *lin-44::gfp* (Murakami et al., 2001) and pBluescript (Stratagene). The concentrations of the injected *ceh-17::ser-6*, *lin-44::gfp*, and pBluescript were 10, 20, and 70 ng/µL, respectively. Animals carrying *lin-44::gfp*, reflected by expression of GFP in the tail hypodermis, were analyzed in the rescue experiments.

Generation of heterozygous mutants and overexpression of *ser-3* and *ser-6*

To generate heterozygous mutant animals, *ser-3(ad1774);ser-6(tm2104);tzIs3* males and *unc-64(e246)III;tzIs3* hermaphrodites, *ser-3(ad1774);unc-64(e246)III;tzIs3* hermaphrodites or *ser-6(tm2104);unc-64(e246)III;tzIs3* hermaphrodites were mated before each assay. *unc-64* homozygous animals exhibit an uncoordinated phenotype (Unc) since *unc-64* gene encodes syntaxin which is required for vesicle release, including neurotransmitter release (Brenner, 1974). Only non-Unc F1 animals were tested as Unc animals result from self-fertilization. These heterozygous animals possessed a mutation in *unc-64* gene, which was used for a mating marker. It should not affect their CREB activities because the mutation is recessive.

To obtain strains that overexpress SER-6 in the SIA neurons, *ceh-17::ser-6* was injected into *ser-3(ad1774);tzIs3*, together with *lin-44::gfp* and pBluescript (Stratagene). The concentration of the injected expression plasmids, *lin-44::gfp*, and pBluescript were 10, 10, and 80 ng/μL, respectively. CREB activation was analyzed using transformants that express GFP in the tail hypodermis.

To obtain strains that overexpress SER-3 in the SIA neurons, the *ceh-17::ser-3* fusion construct (Suo et al., 2006) was injected into *ser-3(ad1774);tzIs3* together with *lin-44::gfp* and pBluescript (Stratagene). The concentrations of the injected expression plasmids, *lin-44::gfp*, and pBluescript were 10, 10, and 80 ng/μL, respectively. The transformant was then mated with *tzIs3* males, and the sibling *tzIs3* animals carrying the *ceh-17::ser-3* fusion construct was mated with *ser-6(tm2104);tzIs3* males to obtain *ser-6(tm2104);tzIs3* carrying the *ceh-17::ser-3* fusion gene.

Constructs for expression in cultured cells

To express SER-3 and SER-6 epitope-tagged at their C-termini in mammalian culture cells, following constructs were generated. The coding sequence of *ser-3* was amplified with the corresponding forward and reverse primers by PfuUltra High-Fidelity DNA Polymerase (Stratagene) using subcloned *ser-3* cDNA as the template. The PCR product was digested with the restriction enzymes *Hind*III and *Apa*I and cloned into *Hind*III- and *Apa*I-digested pcDNA3.1/V5-His (Life Technologies). The coding sequence of *ser-6* was amplified with the corresponding forward and reverse primers by PfuUltra High-Fidelity DNA Polymerase (Stratagene) using subcloned *ser-6* cDNA as the template. The PCR product was digested with the restriction enzymes *Hind*III and *Sal*I and cloned into *Hind*III- and *Sal*I-digested pcDNA3.1/3xHA. Using this expression construct, SER-6 expressed in HEK 293 cells was barely detected by Western blotting. To enhance SER-6 expression in heterologous system, codons of *ser-6* were optimized to human's usage and the optimized *ser-6* was synthesized (Operon). The synthesized *ser-6* was digested with the restriction enzymes *Hind*III and *Xho*I and cloned into *Hind*III- and *Xho*I-digested pcDNA3.1/3xHA. The sequences of primers used for construction of the expression vectors are shown in Table 1.

For the luciferase assay, two luciferase reporter vectors were used: CRE-Luc and AP1-Luc. They consist of response elements and the firefly luciferase site in the modified pGL3 vector (Promega).

Cell culture and transfection of plasmid DNA to HEK 293 cells

HEK 293 cells were cultured in 12 well plates in DMEM containing 10% FBS at $37^{\circ}C$, 5% CO_2 until they reach about 70-90% confluency. Plasmids (1.6 ug in total)

were transfected into the HEK 293 cells using Lipofectamine 2000 reagent (Invitrogen). Forty eight hours later, the transfected cells were collected and washed with ice-cold 1x PBS. Since *ser-6* expressed in HEK 293 cells was barely detected by Western blotting, the proteasome inhibitor MG-132 and the antagonists of amine receptors chlorpromazine and clozapine were used. Ten micromolar MG-132, 10nM chlorpromazine or 10nM clozapine were added to the medium after 24 hours of transfection. Four hours later, the transfected cells were collected and washed with ice-cold 1x PBS.

The collected cells were lysed in buffer containing 150mM NaCl, 20mM Tris-HCl (pH 7.6), 1mM EDTA, 1% Triton X-100 and protease inhibitor cocktail (SIGMA-ALDRICH). The cells were homogenized by sonication (Branson). The cell lysates were left on ice for 20 minutes and then were spun at 13,000 rpm for 30 minutes at $4^{o}C$ by Centrifuge5415 R (Eppendorf). The pellet was dissolved in 1x protein sample buffer containing 0.25 M Tris-HCl (pH 6.8), 12% 2-mercaptoethanol, 4% SDS, 20% glycerol, 0.02% BPB and 8 M urea, then the samples were incubated at room temperature for 30 minutes, at 65°C for 15 minutes or 100°C for 5 minutes. The samples were stored at -20°C until used.

SDS-PAGE and transfer

About 50 μg of protein were loaded in each lane, and were separated by SDS-PAGE on standard stacking and 10% Tris-HCl polyacrylamide running gels with constant current at 25 mA, and transferred to PVDF membranes (Immobilon-P, IPVH00010. Millipore) using the wet tank, TE-22 (Amersham) with constant current at 150 mA for 90 minutes at $4^{o}C$. Membranes were equilibrated with methanol for 30

seconds, MilliQ water for 2 minutes and transfer buffer (195 mM glycine, 25 mM Tris, 20% methanol).

Antibody

Mouse monoclonal anti-HA (1:5000; 12CA5, Roche) and mouse monoclonal anti-V5 (1:5000; P/N 46-0705, Invitrogen) were used as the primary antibodies. HRP-linked ant-mouse IgG (1:5000; #7074, Cell Signaling) was used as the secondary antibody.

Immunoblotting

The membrane was incubated for 1 hour at room temperature in TBST buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20] containing 5% dry skim milk to block excess protein binding sites on the membrane. The membrane was then washed three times, for 5 minutes each, with TBST buffer. The membrane was incubated overnight at 4°C with the primary antibodies diluted in TBST containing 2% ECL advanced blocking agent (GE Healthcare), and was washed with TBST buffer three times for 5 minutes each with TBST buffer. The membrane was then incubated for 1 hour at room temperature with secondary antibody diluted in TBST containing 5% skim milk, and washed with TBST buffer three times for 5 minutes each. ECL prime (GE Healthcare) was used to visualize target protein. The images of protein bands were obtained by the LAS-3000 (FUJI FILM) and analyzed with the software, Multigauge ver. 2.3.

Luciferase assay

The methods used for cell culturing, gene transfection and luciferase activity measurements followed the standard methods of the Dual-Glo Luciferase Assay System (Promega). HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% FBS at 37°C in an atmosphere of 5% CO_2 in 6cm dishes until they grew to 70-90% confluency. The cells were transfected with 2.02 μg of total plasmid using Lipofectamine 2000 reagent (Invitrogen). The luciferase reporter gene (1 μg) and an expression vector (1 μg) or empty vector (1 μg) were co-expressed in the cells. The plasmid pRL-TK (Promega) containing the sea pansy (renilla) luciferase gene was co-transfected (20 ng) as an internal control to normalize the transfection efficiency and number of cells in all experiments. In all cases, total DNA per well was standardized using an empty vector. After 6 hours, the cells were transferred into collagen-coated 96-well plates. After 18 hours of culturing, the medium was discarded. The cells were washed with 1x PBS and then incubated in DMEM containing octopamine but not FBS for 3 hours.

Luciferase activity was determined using the Dual-Glo Luciferase Assay System (Promega). Luciferase activity was measured by a luminometer (Centro LB 960, Berthold) for 10 seconds after a 2 seconds delay. Luciferase activities were calculated as follow: relative light unit = [values for luminescence of firefly luciferase] / [values for luminescence of renilla luciferase].

Strains, Cloning of *ser-6,* Phylogenetic analysis, Analysis of CRE-mediated gene expression, Analysis of *ser-6* expression patterns, Cell-specific rescue of *ser-6* and Generation of heterozygous mutants and overexpression of *ser-3* and *ser-6* are the

Results

SER-6 is highly homologous to the octopamine receptor SER-3

To investigate the involvement of SER-6 in octopamine-mediated CREB activation, I extracted total RNA from wild-type N2 animals and cloned cDNA of the *ser-6* gene using a *ser-6* reverse primer and an SL1 primer matching the 5'-trans-spliced leader sequence found on *C. elegans* RNAs. In *C. elegans,* the 5' ends of pre-mRNAs are trimmed off and replaced with SL1 sequence. More than half of *C. elegans* pre-mRNAs are subject to SL1 trans-splicing (Blumenthal, 2005). The resulting PCR product was electrophoresed and only a single band was observed, suggesting that SER-6 does not have splice variants (Fig.2 A). cDNA of *ser-6* was then cloned into an expression vector. Splicing pattern of *ser-6* which was estimated from its genome sequence is available on Wormbase, an online database of the genome of *C. elegans* (Fig.2 B). In order to identify its sequence, the cloned *ser-6* cDNA was sequenced and it was found that the fifth exon of predicted *ser-6* is not included in the cDNA (Fig.2 C). I then compared the amino acid sequence of SER-6 to that of SER-3 (Fig.2 D) and showed that SER-3 and SER-6 are highly homologous. The phylogenic tree including human and invertebrate amine receptors (Fig.2 E) shows that SER-6 is homologous to other Gq-coupled octopamine receptors of invertebrates, including SER-3 and insect octopamine receptors AmOAMB and DmOAMB (Han et al., 1998; Grohmann et al., 2003). Among mammalian amine receptors, SER-6 was most closely related to the human α1-adrenergic receptors, which are also Gq-coupled receptors.

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SER-6 is involved in octopamine-dependent CREB activation in the SIA neurons

In *C. elegans*, CREB activation can be detected by fluorescence in animals carrying a *cre::gfp* reporter, in which CRE is fused to a GFP sequence (Kimura et al., 2002). Using this reporter, it has been shown that food deprivation induces CREB activation in the SIA neurons (Suo et al., 2006; Suo et al., 2009). This response appears to be mediated through octopamine, since exogenously applied octopamine similarly activates CREB in the SIA neurons, and mutants in the *tbh-1* gene, which encodes a tyramine β hydroxylase required for octopamine synthesis (Alkema et al., 2005), exhibit decreased response to food deprivation. SER-3 has been shown to function in the SIA neurons to transmit octopamine signaling through EGL-30, the α subunit of Gq (Fig.1). Here, I determined whether SER-6 is also involved in this CREB activation.

Animals carrying *cre::gfp* were exposed to 3 mg/mL octopamine for 4 hours, or deprived of food for 6 hours. The number of SIA neurons in each animal expressing GFP was then counted to quantify CREB activation. Wild-type animals exhibited significant GFP expression in the SIA neurons following octopamine treatment or food deprivation (Fig.3 B,E). *C. elegans* has four equivalent SIA neurons (SIADL, SIADR, SIAVL, and SIAVR) and there was no apparent difference in GFP expression rates of these four neurons. As reported previously, this CRE-mediated gene expression was dependent on SER-3, with *ser-3* mutants showing decreased responses to exogenous octopamine and food deprivation (Fig.3 F). Next, I examined two deletion alleles of *ser-6*, *tm2104* and *tm2146*, and found that octopamine-mediated GFP expression was decreased in both mutants (Fig.3 D,G,H). These results suggest that SER-6 is also required for octopamine-dependent CREB activation in the SIA neurons. CREB activation levels induced by food deprivation were also decreased in *ser-6* mutant

animals (Fig.3 G,H), suggesting that SER-6 is involved in food deprivation-induced CREB activation in the SIA neurons.

In octopamine-deficient *tbh-1* mutants the response to food deprivation was significantly attenuated (Fig.3 J). However, a small response was observed, consistent with previous reports (Suo et al., 2006), suggesting that the response to food deprivation is partially octopamine-independent. The level of CREB activation observed in the *ser-3* mutants in the absence of food was similar to that of *tbh-1*. I also analyzed *tbh-1;ser-3* double mutants and found that *tbh-1;ser-3* responded to food deprivation slightly stronger than *ser-3* and *tbh-1* single mutants (Fig.3 K). The reason for this is unknown. However, since CREB activation was not decreased by the *tbh-1* mutation in the double mutants, it is likely that CREB activity observed in the *ser-3* mutants is octopamine-independent. In contrast, the level of CREB activation in the *ser-6* mutants was higher than that in the *tbh-1* mutants, and the level of CREB activation in the *tbh-1;ser-6* mutants was similar to that in the *tbh-1* mutants (Fig.3 L). These results suggest that some octopamine-dependent signaling is occurring in the absence of *ser-6*. These experiments were repeated in *ser-3;ser-6* double mutants, and their responses to exogenous octopamine and food deprivation were similar to that of the *ser-3* mutants (Fig.3 I).

When animals are soaked in water, CREB is activated in the SIA neurons, and this soaking response is independent of octopamine (Suo et al., 2006). *ser-6* mutants responded normally to soaking (Fig.3 G), exhibiting robust activation of CREB. This result confirms that the SIA neurons are present in *ser-6* mutants and that CREB can be activated in these neurons under certain conditions. Reduced octopamine-mediated CREB activation seen in the *ser-6* mutants is therefore not the result of abnormal

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development of SIA neurons.

Previously, Suo et al. showed that in the presence of food dopamine signal inhibits CREB activation in SIA neurons since exogenous dopamine treatment caused inhibition of CREB activation in the presence of exogenous octopamine and CREB was spontaneously activated in the presence of food in the dopamine-deficient mutant *cat-2* (Suo et al., 2009; Fig.1). Although response to exogenous octopamine was examined in the presence of food, wild-type animals responded strongly to exogenous octopamine (Fig.3 B,E). It is likely that the amount of exogenous octopamine is much greater than that of endogenous dopamine and therefore exogenous octopamine was able to activate CREB even in the presence of suppression by dopamine signaling. *ser-3* mutants and *ser-6* mutants did not respond to exogenous octopamine (Fig.3 F,G,H). It is possible that dopamine signal induced by food inhibited the effect of exogenous octopamine when *ser-3* or *ser-6* is absent. I therefore examined the effect of endogenous dopamine in *ser-3* and *ser-6* background using dopamine and octopamine-deficient mutant *cat-2;tbh-1*. As mentioned above, in *cat-2* mutants, CREB is spontaneously activated in the presence of food by endogenous octopamine. Therefore, to observe an increase in the level of CREB activation, *cat-2;tbh-1* mutants were used to reduce the basal level of CREB activation. In *cat-2;tbh-1;ser-3* mutants and *cat-2;tbh-1;ser-6* mutants the levels of CREB activation were similar to that of *cat-2;tbh-1* mutants and there was no octopamine-dependent CREB activation (Fig.4; Suo et al., 2009), suggesting that exogenous octopamine cannot activate CREB in *ser-3* mutants and *ser-6* mutants even in the absence of endogenous dopamine.

In addition to SER-3 and SER-6, the *C. elegans* genome contains another octopamine receptor, OCTR-1. The tyramine receptor TYRA-3 also has been shown to

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bind octopamine, albeit weakly. I therefore investigated whether OCTR-1 and TYRA-3 are involved in octopamine-mediated CREB activation. The *octr-1* and *tyra-3* mutants responded normally to exogenous octopamine and food deprivation, suggesting that these receptors are not involved in the octopamine-mediated CREB activation seen in the SIA neurons (Fig.3 M,N).

SER-6 functions in the SIA neurons to activate CREB

The observation that octopamine-induced CREB activation was reduced in both *ser-3* and *ser-6* single mutants indicates that both SER-3 and SER-6 are required for CREB activation. Furthermore the observation that the response to food deprivation in *ser-3;ser-6* double mutants was not smaller than that of either *ser-3* or *ser-6* single mutants also suggests that SER-3 and SER-6 are not redundant. One possibility is that they function in different neurons. Notably, it has been shown that both SER-3 and SER-6 are required for regulation of octanol sensitivity by octopamine and that they function in different neurons for this regulation (Mills et al., 2012). Another possibility is that SER-3 and SER-6 function in the same (SIA) neurons and there may be some interaction at the molecular level. It has been previously reported that *ser-6* is expressed in a subset of head and tail neurons (Srinivasan et al., 2008). However, it has not been determined whether *ser-6* is expressed in the SIA neurons. I generated a *ser-6::gfp* reporter fusion gene in which 5 kb of upstream sequence plus a portion of exon 1 were fused to the *gfp* gene (Fig.5). This fusion gene was co-injected along with the *ceh-17::dsred* reporter. The *ceh-17* promoter was used as it induces gene expression in only the four SIA neurons and one additional neuron (the ALA neuron) (Pujol et al., 2000). The *ceh-17::dsred* reporter therefore labels the SIA neurons with DsRed

expression. A *tbh-1::dsred* reporter construct was also introduced to label the octopaminergic RIC neurons. In these transformants, GFP expression was observed in multiple neurons, with GFP colocalizing with DsRed (Fig.6), suggesting that *ser-6* is expressed in both the SIA and RIC neurons.

I then performed a cell-specific rescue experiment to determine whether SER-6 functions in the SIA neurons. I introduced the *ceh-17::ser-6* fusion construct, in which the *ceh-17* promoter was fused to SER-6 cDNA, into *ser-6(tm2104)* mutant animals. These transformants should express SER-6 in only the SIA and ALA neurons. As shown in Fig.7, the transgenic animals responded to exogenous octopamine as robustly as did the wild-type animals, suggesting that expression of SER-6 in the SIA neurons is sufficient to restore CREB activation upon octopamine. CREB activation of the transformants in response to food deprivation was not significantly different from the wild-type animals, also suggesting that SER-6 functions in the SIA neurons for food deprivation response. However, there was no significant difference between CREB activation levels for food deprivation of *ser-6* animals and the transformants. Therefore, it remains possible that *ser-6* also functions in other cells in addition to the SIA neurons.

Both SER-3 and SER-6 are required for normal CREB activation in SIA neurons

The above results suggest that SER-3 and SER-6 function in the same cells, and that both of these receptors are required for normal signaling, despite having similar function. One explanation for the decreased CREB activation seen in *ser-3* and *ser-6* single mutants is a decrease in the total number of octopamine receptors. A specific level of octopamine receptor may be required for normal signaling, and removal of either of these two genes results in an insufficient quantity of octopamine receptors. To

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address this possibility, I assayed CREB activation in double heterozygous *ser-3/+;ser-6/+* animals. The double heterozygous animals responded slightly weaker to exogenous octopamine treatment than wild-type animals (Fig.8 B). However, the response of the double heterozygous animals were much stronger than that of the *ser-3* or *ser-6* single mutants, which was essentially zero (Fig.3 F,G,H). This result suggests that having both *ser-3* and *ser-6* is important for CREB activation rather than the total quantity of octopamine receptor genes. The response to food deprivation was not different between *ser-3/+;ser-6/+* double heterozygous animals and wild-type animals (Fig.8 A,B). Furthermore, I analyzed the *ser-3/ser-3;ser-6/+* and *ser-3/+;ser-6/ser-6* heterozygous animals and found that *ser-3/ser-3;ser-6/+* were similar to *ser-3* single mutants (P>0.05, Fig.3 F, Fig.8 C) and that *ser-3/+;ser-6/ser-6* were similar to *ser-6* single mutants (P>0.05, Fig.3 G, Fig.8 D) with respect to their response to food deprivation. These results suggest that removing one copy of the *ser-3* or *ser-6* gene have little effect on the response to food deprivation, which further supports the idea that normal CREB activation requires the existence of both octopamine receptors rather than just a specific quantity of receptor.

To further address the effect of the gene dosage, I next assessed CREB activation in animals overexpressing either SER-3 or SER-6. SER-3 was overexpressed in the SIA neurons of the *ser-6* deletion mutant using the *ceh-17::ser-3* fusion construct, while SER-6 was overexpressed in the SIA neurons of *ser-3* deletion mutant using the *ceh-17::ser-6* fusion construct. These animals therefore lacked either SER-6 or SER-3, but overexpressed the other receptor in the SIA neurons, in addition to endogenous expression. It has been shown that multiple copies (typically over 100 copies) of genes are retained in transgenic animals when transformed by injection (Fire et al., 1991). In

ser-3 mutants overexpressing SER-6, CREB activation induced by exogenous octopamine or food deprivation was similar to that for *ser-3* deletion mutants alone (P>0.05, Fig.3 F, Fig.8 E). This result suggests that SER-6 alone cannot induce activation of CREB, even when SER-6 is overexpressed. In *ser-6* mutants overexpressing SER-3, some spontaneous CREB activation was observed on the control plates that did not contain octopamine but did contain food (Fig.8 F, first bar). However, this activation was not seen on NGM plates containing food (Fig.8 F, third bar); the cause of this difference is unknown. One possible explanation is that because control plates for octopamine treatment contained less salts and peptone than NGM plates, these compounds, or the difference in the condition of the bacteria growing on these plates, may have affected CREB activation in this strain. Nonetheless, a moderate increase in CREB activation was observed upon exogenous octopamine treatment in the *ser-3*-overexpressing animals (Fig.8 F), suggesting that SER-3 can partially respond to exogenous octopamine without SER-6 when overexpressed. In contrast, the level of CREB activation induced by food deprivation in *ser-3*-overexpressing animals was not different from that of *ser-6* mutants (P>0.05). Collectively, these results suggest that both *ser-3* and *ser-6* are required for full activation of CREB regardless of their quantity and that *ser-3* but not *ser-6* can partially function by itself only when it is overexpressed.

Heterologous expression of SER-3 and SER-6

To investigate how *ser-3* and *ser-6* function to activate CREB, SER-3 and SER-6 was heterologously expressed in human embryonic kidney cells, HEK 293 cells. First, Western blotting was performed to examine the expression of V5-tagged SER-3 and

HA-tagged SER-6 in HEK 293 cells. As shown in Fig.9, V5-tagged SER-3 was detected. When samples were incubated at 100°C for 5 minutes in the sample buffer, SER-3 remained in stacking gel. When the samples were incubated at room temperature or 65°C, SER-3 was detected at lower position of the membrane possibly forming oligomers. SER-3 may have remained in stacking gel since it aggregated as it is common for hydrophobic proteins to aggregate when incubated at 100°C. HA-tagged SER-6 was barely detected by Western blotting (Fig.10). SER-6 might be degraded immediately after translation. Therefore, the proteasome inhibitor MG-132 was added to the medium. The antagonists of amine receptors chlorpromazine and clozapine were also added to the medium, since they could function as pharmaceutical chaperons which stabilize the conformation of SER-6 to prevent degradation. However, a treatment with MG-132, chlorpromazine or clozapine did not have any influence on the expression of SER-6. To enhance SER-6 expression in heterologous system, codons of *ser-6* were optimized for human's usage (Fig.11). The optimized version of *ser-6* cDNA was synthesized by Operon and was subcloned into an expression vector. SER-6 was expressed more efficiently when its codons were optimized to the human codon usage. MG-132, chlorpromazine, and clozapine did not have any effect on the expression level of optimized SER-6 (Fig.10).

Next, octopamine induced-intracellular signaling transduced by SER-3 and SER-6 in HEK 293 cells was analyzed. Upon stimulation, Gs-coupled receptors activate adenylyl cyclase resulting in an increase of cAMP, and Gq-coupled receptors activate phospholipase C resulting in an increase of intracellular Ca^{2+} concentration. Luciferase reporter genes, CRE-Luc and AP1-Luc contain CRE or AP1 response element fused to luciferase gene. These reporter genes allow for indirect measurement of cAMP

production and intracellular Ca^{2+} mobilizations, respectively, by expressing luciferase in response to the signal transduced by GPCRs (Cheng et al., 2010). The expression vectors of V5-tagged SER-3, HA-tagged SER-6 (optimized to human codon usage) or both, and CRE-Luc or AP1-Luc were transfected to HEK 293 cells along with the renilla luciferase vector, and exogenous octopamine was added to medium. Three hours later, luciferase activities in each condition were determined. The renilla luciferase vector was co-transfected as an internal control to normalize the transfection efficiency and number of cells in all experiments. In HEK 293 cells transfected with an empty vector or the *ser-6* expression vector and AP1-Luc, octopamine did not induce dose-dependent expression of luciferase whereas activation of SER-3 by octopamine showed dose-dependent response. Furthermore, cells co-expressing SER-3 and SER-6 responded to octopamine as strong as SER-3 expressing cells in AP1-reporter assay (Fig.12 A). Similarly, cells expressing SER-3 responded to octopamine in dose dependent manner in CRE-reporter assay but not in cells expressing SER-6. Cells expressing SER-3 and SER-6 responded to octopamine as strongly as cells expressing SER-3 (Fig.12 B). These results suggest that SER-3, but not SER-6, activates Gq and Gs signal upon octopamine stimulation, and SER-6 did not enhance Gs and Gq signaling transduced by SER-3.

To understand whether SER-3 activates Gs signal, further experiments is required since α adrenergic-like octopamine receptors lead to the elevation of both Ca^{2+} and cAMP in response to octopamine when expressed in mammalian cultured cells, although physiological relevance of this function is unclear. Previously, SER-6 has been shown to be a Gq-coupled octopamine receptor using a heterologous expression in *Xenopus* oocytes (Mills et al., 2012). Therefore, functional SER-6 might not be

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expressed in HEK 293 cells and further investigation is needed to explore the function of SER-6.

Texts on p.26, l.2 - p.33, l.20 are the pre-peer reviewed version of the following article: Yoshida M, Oami E, Wang M, Ishiura S, Suo S. 2014. Nonredundant function of two highly homologous octopamine receptors in food-deprivation-mediated signaling in *Caenorhabditis elegans*. J Neurosci Res., which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1002/jnr.23345/full].

Discussion

Neurotransmitters commonly possess multiple receptors that couple to the same intracellular signaling. When expressed in a heterologous system, such similar receptors function in a non-redundant manner through receptor-receptor interactions. In the present thesis, I analyzed two homologous octopamine receptors of *C. elegans*, SER-3 and SER-6, which have been shown to be coupled to the same class of G proteins (Petrascheck et al., 2007; Mills et al., 2012). First, I cloned cDNA of *ser-6* and compared the amino acid sequence of SER-6 to that of SER-3. As expected, SER-3 and SER-6 were highly homologous to each other. Next, I analyzed whether SER-6 is involved in octopamine-mediated CREB activation, similar to SER-3. As seen in *ser-3* deletion mutants, octopamine- and food deprivation-mediated CREB activation were decreased in *ser-6* deletion mutants compared to wild-type animals, suggesting that both SER-3 and SER-6 are required for signal transduction. Cell-specific expression of SER-6 in the SIA neurons was sufficient to restore CREB activation in the *ser-6* mutants, indicating that SER-6 functions in SIA neurons. Moreover, *ser-3/+;ser-6/+* double heterozygous animals responded strongly to exogenous octopamine and food deprivation. *ser-3* mutants overexpressing SER-6 responded to exogenous octopamine and food deprivation as weakly as *ser-3* mutants. Furthermore, overexpressed SER-3 in *ser-6* mutants did not fully rescue CREB activation. Taken together, these results suggest that SER-3 and SER-6 act non-redundantly to transmit octopamine signaling in the SIA neurons (Fig.13).

Using SER-3 and SER-6 overexpressing animals, I further demonstrated that both SER-3 and SER-6 are required for normal CREB activation by octopamine, as

overexpression of one receptor in the absence of the other could not fully restore normal CREB activation. To further support the non- redundant function of *ser-3* and *ser-6*, the amounts of SER-3 and SER-6 expressed in the SIA neurons need to be determined for example by Western blotting of SIA neurons with anti-SER-3 antibody and anti-SER-6 antibody. *ser-3*-overexpressing animals did respond to exogenous octopamine in the absence of *ser-6*, though the response was much weaker than that in the wild-type animals. In contrast, SER-6 could not activate CREB without SER-3 even when overexpressed. These results indicate that, when overexpressed, SER-3 can partially bypass the requirement of SER-6. In addition, CREB activation by food deprivation was stronger in *ser-6* mutants than in *ser-3* mutants or *ser-3;ser-6* double mutants (Fig.3), suggesting that SER-3 can also partially activate CREB without SER-6 in this condition. One possible mechanism in which SER-3 and SER-6 function non-redundantly is that SER-6 functions in part to assist the function of SER-3 by controlling the quantity of functional SER-3. For example, SER-6 might enhance the efficiency of cell surface expression of SER-3 in a similar manner as α_{1b}-adrenergic receptor and α_{1d}-adrenergic receptor, homolog of SER-3 and SER-6, forming a heterodimer to affect their membrane expression (Stanasila et al., 2003; Hague et al., 2004). Another possibility is that SER-3 and SER-6 form a dimer and that the heterodimer transmit stronger signals than monomers or homodimers; it has also been shown that α_{1b}-adrenergic receptor and α_{1d}-adrenergic receptor affects their signaling strength by forming heterodimer (Hague et al., 2006).

The octopamine-containing plate used in this study was covered with bacterial food, which induce dopamine signal that inhibits CREB activation (Suo et al., 2009). CREB in SIA neurons was not activated by exogenous octopamine in *ser-3* mutants and

ser-6 mutants, but it was in wild-type animals (Fig.3 B,D-H). From these results, it is possible that, in wild-type animals, excess octopamine signaling mediated by exogenous application of octopamine overcomes suppression by endogenous dopamine singling and that, for some reason, both *ser-3* and *ser-6* is required for activating CREB in the presence of inhibitory dopamine signaling. However, *cat-2;tbh-1;ser-3* mutants and *cat-2;tbh-1;ser-6* mutants did not respond to exogenous octopamine (Fig.4), suggesting that even in the absence of suppression by dopamine signaling, *ser-3* or *ser-6* single mutants cannot activate CREB. Therefore, it is unlikely that suppression by dopamine signaling plays a role in the non-redundant function of *ser-3* and *ser-6*. It also remains possible that even though SER-3 and SER-6 are structurally similar, they transmit different intracellular signals *in vivo* and these signals converge to fully activate CREB. Further efforts, including expression of SER-3 and SER-6 in a heterologous expression system, would be required to elucidate the precise mechanisms by which these receptors function non-redundantly.

I found that SER-3 and SER-6 are co-expressed in the SIA neurons. While both SER-3 and SER-6 are also expressed in other neurons, the expression patterns of these receptors overlap only partially. Neurons expressing only SER-3 or SER-6 are unlikely to be able to respond to octopamine stimulation by fully activating CREB, unlike the SIA neurons. It is therefore possible that, by utilizing multiple functionally similar receptors differentially expressed across several cells types, the nervous system diversifies its sensitivity to neurotransmitters, allowing for more complex neuronal regulation.

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Wang M, Ishiura S, Suo S. 2014. Nonredundant function of two highly homologous octopamine receptors in food-deprivation-mediated signaling in *Caenorhabditis elegans*. J Neurosci Res., which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1002/jnr.23345/full].

Conclusion

In the present thesis, I analyzed two highly homologous octopamine receptors of *C. elegans* SER-3 and SER-6. I found that *ser-6* is also required for food-deprivation induced CREB activation in the cholinergic SIA neurons as *ser-3* does since CREB activation induced by exogenous octopamine and food deprivation was decreased in *ser-6* deletion mutants and expression of SER-6 in the SIA neurons was sufficient to restore CREB activation in the *ser-6* mutants. Furthermore, I also demonstrated through genetic experiments that both of these receptors function in the same cells in a non-redundant manner since the response to exogenous octopamine and food deprivation was not different between *ser-3/+;ser-6/+* double heterozygous animals and wild-type animals, *ser-3* mutants overexpressing SER-6 responded as weakly as *ser-3* mutants, and overexpressed SER-3 in *ser-6* mutants did not fully restore CREB activation.
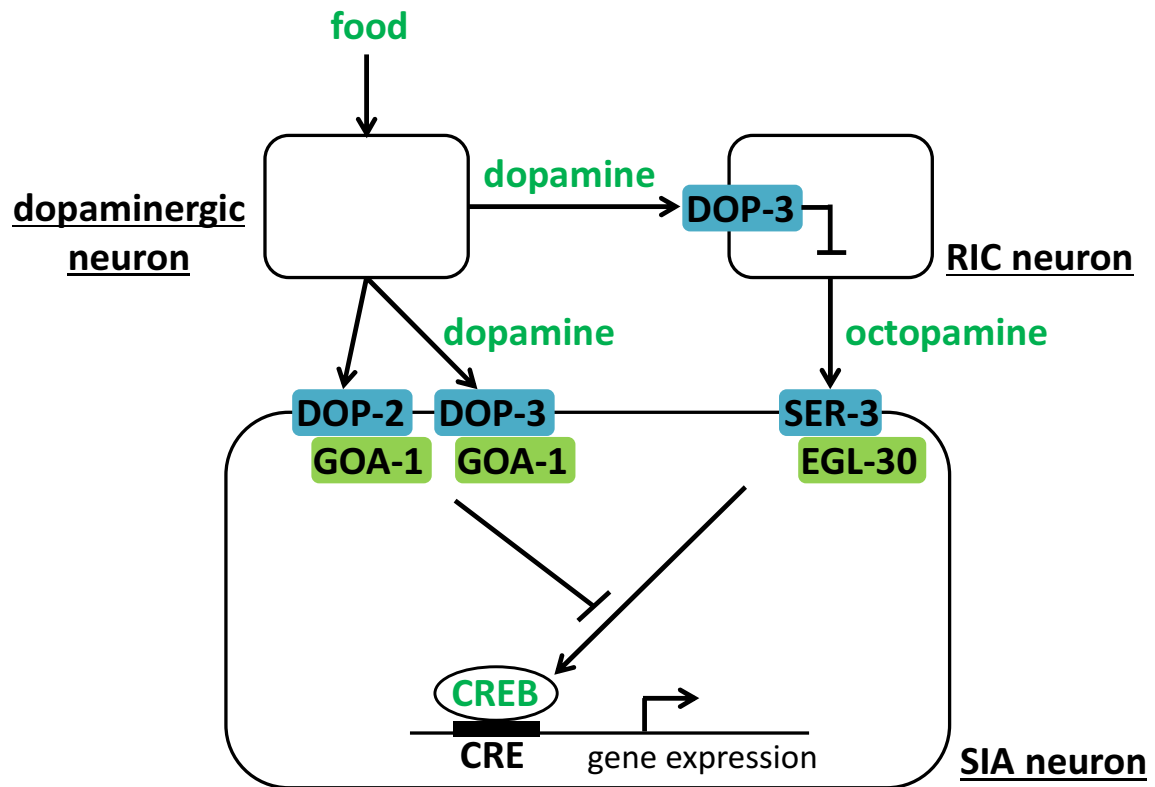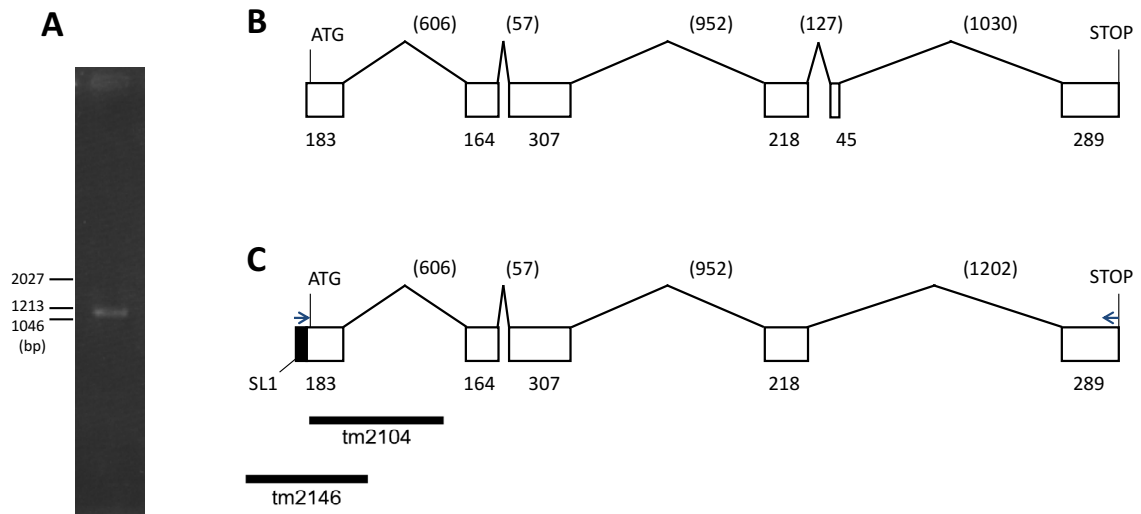
Fig.1   Model for food deprivation-induced CREB activation regulated by dopamine and octopamine.

During food deprivation, dopamine is not released, and octopamine, which is released from the octopaminergic RIC neurons, activates the octopamine receptor SER-3 in the SIA neurons. SER-3 works through EGL-30 (Gqα) to activate CREB. In the presence of food, dopamine is released and suppresses octopamine release through the dopamine receptor DOP-3 in the RIC neurons. In addition, dopamine inhibits Gq signaling in the SIA neurons by activating GOA-1 (Goα) through D2-like dopamine receptors DOP-2 and DOP-3 to suppress CREB activation in the SIA neurons. (Modified from Suo et al., 2009)

A

2027 —
1213 —
1046 —
(bp)

B

ATG (606) (57) (952) (127) (1030) STOP

183   164 307   218 45   289

C

ATG (606) (57) (952) (1202) STOP

SL1 183   164 307   218   289

tm2104

tm2146

D

```
            Nt           TM I                            TM II
SER-6 ( 1)-ILLSLTLGVVNCFVVIGNLLVFYVILTKKSLQTSTNHLVLSLTISDLLLGILILPFAIIQEHSN-EWIFGHFGCRLWLSVDVFLS
SER-3 (17)-FSIISVLTVLVVVVLGNALVIAAVLRRRLRSATGLLILSLALADLLVGTVILPFSIANEVLDQYWIFGETWCTIWLTLDIWMC

        TM III                          TM IV          e2         TM V
SER-6 TASIYNLLAISFDRYMAVRQPIKYPIISSTKVVRLMTFLVWFCSLLLAVILFVLL-(18)-PSMYIIFSAMASFIVPAFVMVILNVRIF
SER-3 TASIYNLVAISIDRYIAIIKPLNYPMLVTKFRARCTVAIVWIGSFLICSPSFFLA-(17)-GRVYVVFSASSSFYIPMIIVFVYFRIY

        i3           TM VI                            TM VII          Ct
SER-6 QTV-(112)-EIRVARTTGIVVAAFIVCWIPFTTIYVLQAYAVCTVAAGCIPASLFTTAFWLGYSNSAVNPILYAAFSRDFR-(13)
SER-3 VAA-(234)-EIKAAKTVAIVTGCFIFCWLGFALVYGLEIKLN---------DVVWSIVFWLGYLNSALNPVIYTVFNREFR-(63)
```
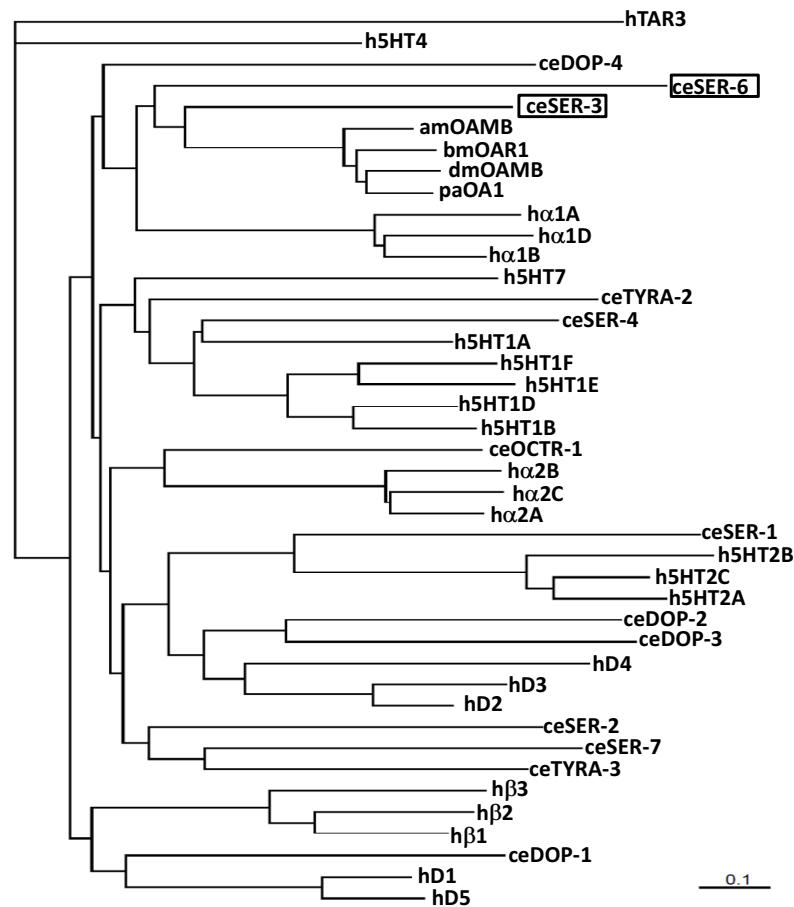
E

Fig.2   Molecular cloning of *ser-6* gene and comparison between SER-6 and other amine receptors.

The cDNA of SER-6 was synthesized using total RNA of *C. elegans* wild-type Bristol N2 as the template and a gene-specific primer. PCR was carried out using cDNA of *ser-6*, a *ser-6* reverse primer and an SL1 primer. Arrows represent the regions which match these primers (C). The resulting PCR product was electrophoresed and a single band was observed (A). *ser-6* cDNA was then sequenced and the structure of this gene was identified (C). B represents the gene structure of *ser-6* which was estimated from its genome sequence on an online database (B). Black bars indicate the region deleted in the *tm2104* and *tm2146* alleles (C). Numbers represent the size of exons and introns (B,C). The amino acid sequence of SER-6 was aligned with SER-3 (D). Predicted transmembrane (TMs) regions are overlined. Amino acid residues conserved between SER-6 and SER-3 are indicated by gray shading. Numbers in parentheses represent the numbers of amino acids not shown in the figure. According to the phylogenic tree of

43

SER-6 and other biogenic amine receptors of human and invertebrates, SER-3 and SER-6 are highly homologous (E). The amino acid sequences of each receptor were aligned with ClustalW using relatively conserved regions, excluding the N terminus, second extracellular loop, third intracellular loop, and the C terminus. The phylogenic tree was calculated using the PHYLIP package using the Fitch-Margoliash method. Receptor sequences used are as follows: *C. elegans* octopamine receptors (ceSER-3 and ceOCTR-1), *C. elegans* dopamine receptors (ceDOP-1, ceDOP-2, ceDOP-3 and ceDOP-4), *C. elegans* tyramine receptors (ceTYRA-2, ceTYRA-3 and ceSER-2), *C. elegans* serotonin receptors (ceSER-1, ceSER-4 and ceSER-7), insect α-adrenergic-like octopamine receptors (dmOAMB, amOAMB, paOA1 and bmOAR1), human dopamine receptors (hD1, hD2, hD3, hD4 and hD5), human serotonin receptors (h5HT1a, h5HT1b, h5HT1d, h5HT1e, h5HT1f, h5HT2a, h5HT2b, h5HT2c, h5HT4 and h5HT7), and human adrenergic receptors (hα1A, hα1B, hα1D, hα2A, hα2B, hα2C, hβ1, hβ2 and hβ3). A human trace amine receptor 3 (hTAR3) was used as an out group. bm: *Bombyx mori*, pa: *Periplaneta americana*, dm: *Drosophila melanogaster,* am: *Apis mellifera.*
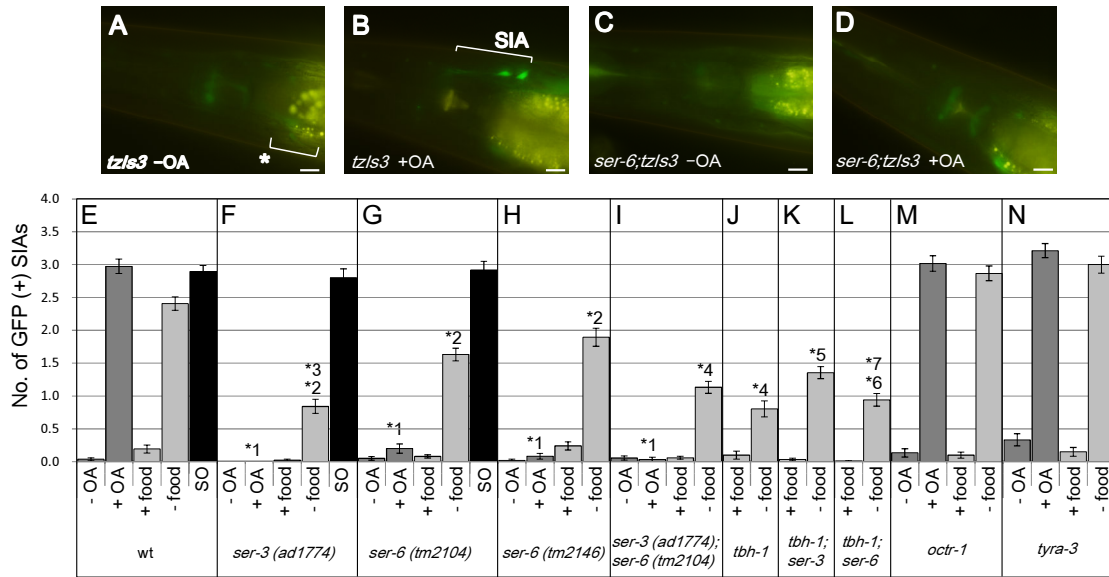
Fig.3 Octopamine and food deprivation-induced CREB activation in the SIA neurons.

Animals carrying *cre::gfp* were cultured on agar plates containing 0 (A, C) or 3 mg/mL (B, D) octopamine. Fluorescent images were obtained from wild-type animals (A, B) and *ser-6(tm2104)* mutants (C, D) after 4 hours of incubation. The bracket marked with an asterisk indicates autofluorescence of the intestine. Each bar represents 10 μm. wild-type, *ser-3(ad1774)*, *ser-6(tm2104), ser-6(tm2146)*, *ser-3(ad1774);ser-6(tm2104)*, *tbh-1(ok1196)*, *ser-3(ad1774);tbh-1(ok1196)*, *ser-6(tm2104);tbh-1(ok1196)*, *octr-1(ok371)* and *tyra-3(ok325)* mutants carrying *cre::gfp* were incubated on plates containing 0 or 3 mg/mL octopamine (OA) for 4 hours, on NGM plates with or without food for 6 hours, or soaked in water (SO) in the presence of food for 4 hours. The number of GFP-expressing SIA neurons per animal was then determined (E-N). Error bars indicate the standard errors of the mean. At least 53 animals were tested. *1:P<0.001 (Tukey–Kramer multiple comparison test), compared with +OA of wild-type animals. *2:P<0.001, compared with -food of wild-type animals. *3:P<0.001, compared with -food of *ser-6(tm2104)* mutants. *4:P>0.05, compared with -food of *ser-3* mutants. *5:P<0.001, compared with –food of *tbh-1* mutants and *ser-3* mutants. *6:P>0.05, compared with –food of *tbh-1* mutants. *7:P<0.001, compared with –food of *ser-6(tm2104)* mutants.
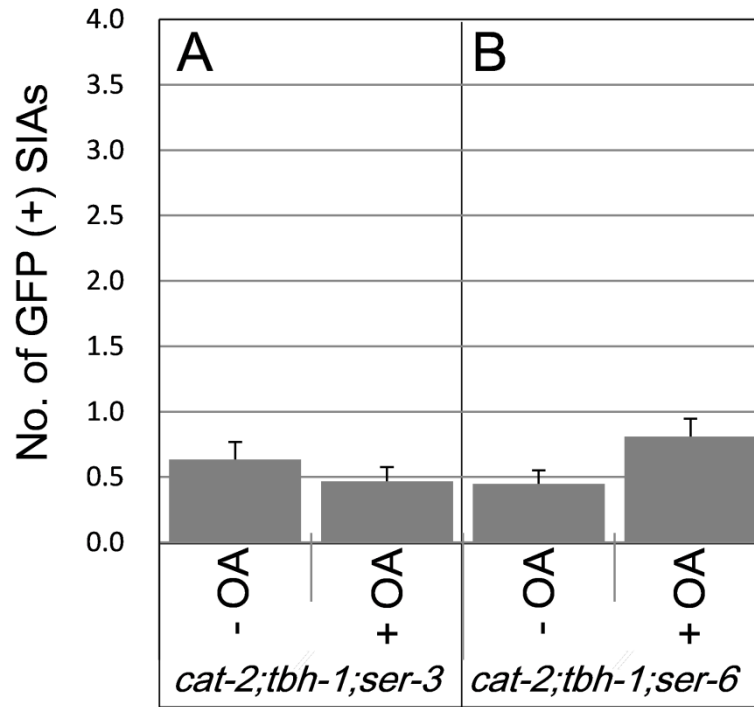
Fig.4 Octopamine-induced CREB activation in the *cat-2;tbh-1* background.
cat-2(e1112);tbh-1(ok1196);ser-3(ad1774) and *cat-2(e1112);tbh-1(ok1196);ser-6 -(tm2104)* mutants carrying *cre::gfp* were incubated on plates containing 0 or 3 mg/mL octopamine (OA) for 4 hours. The number of GFP-expressing SIA neurons per animal was then determined (A,B). Error bars indicate the standard errors of the mean. At least 47 animals were tested. GFP expression was not induced by exogenous octopamine in the SIA neurons of these mutants, and the activation levels in these mutants were similar to that of *tbh-1* mutants.
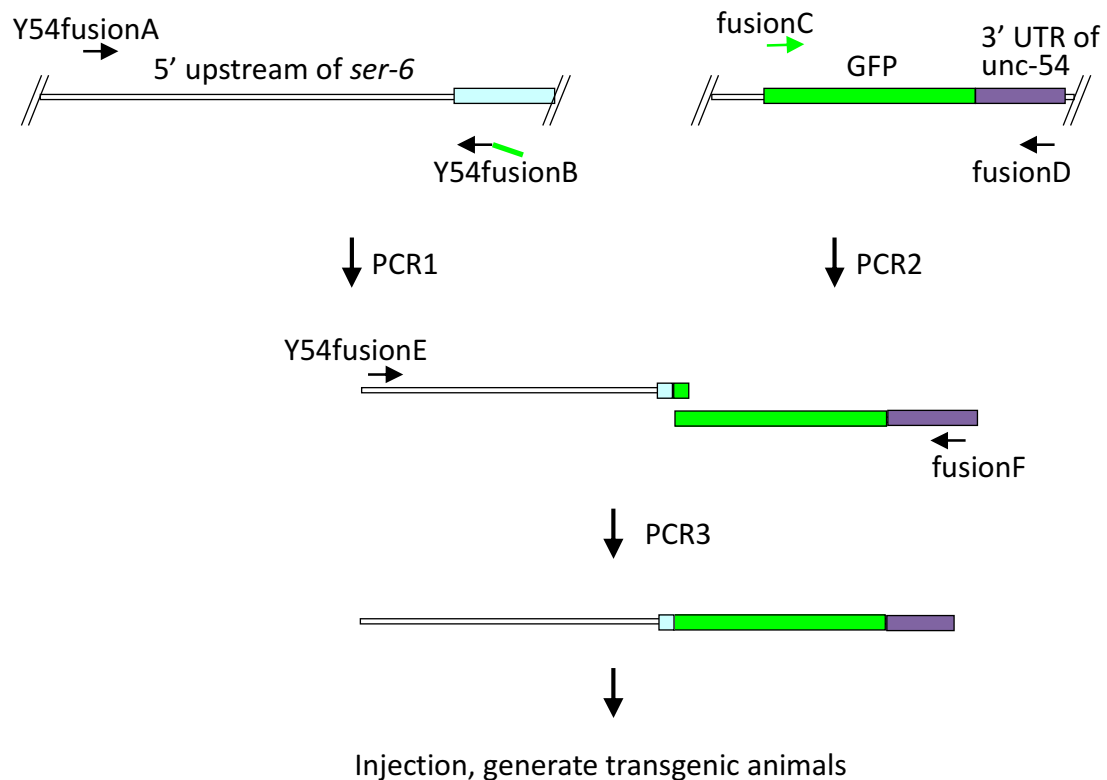
Fig.5　Fusion PCR to generate *gfp* reporter.

To express GFP protein in the cells that express the *ser-6* gene, the *gfp* gene was fused to 5' upstream region of *ser-6* coding sequence that probably contains promoter region of *ser-6*. First, the region corresponding to 5.0-kb upstream and a part of exon 1 of *ser-6* gene (represented by light blue box) was amplified with the primers Y54fusionA and Y54fusionB using genomic DNA as the template (PCR1). The primer Y54fusionB contains overhanging which is complementary to the primer fusionC. The region corresponding to the *gfp* gene and 3'-UTR of *unc-54* (2-1876) (represented by green and purple boxes) were amplified with the primers fusionC and fusionD using pPD95.75 as the template (PCR2). Second, using these PCR products and nested primers Y54fusionE and fusionF, 2nd PCR was performed and *ser-6* promoter fused to *gfp* gene was obtained (*ser-6::gfp*, PCR3). Finally, *ser-6::gfp* was injected to N2 wild-type animals to generate transformants.
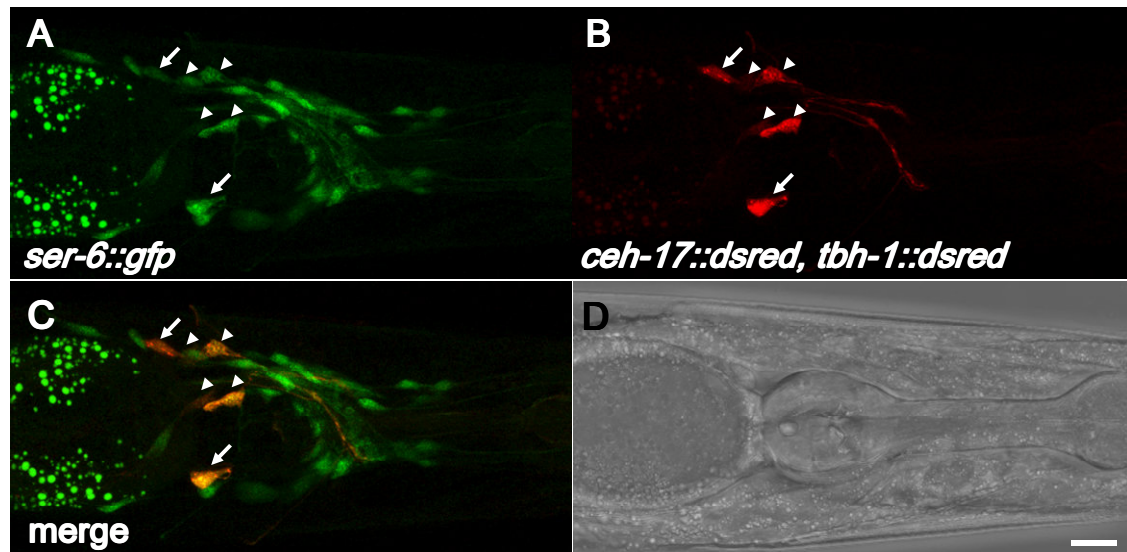
Fig.6　Expression pattern of *ser-6*.

Fluorescent (A, B and C) and corresponding differential interference contrast (D) images were obtained from N2 animals carrying the *ser-6::gfp*, *ceh-17::dsred*, and *tbh-1::dsred* constructs. The SIA- and RIC-neuron-specific promoters, *ceh-17* and *tbh-1*, respectively, were used to label the SIA and RIC neurons with DsRed. Merged images show the colocalization of GFP and DsRed. The bar represents 10 μm. Arrowheads indicate SIA neurons. Arrows indicate RIC neurons.
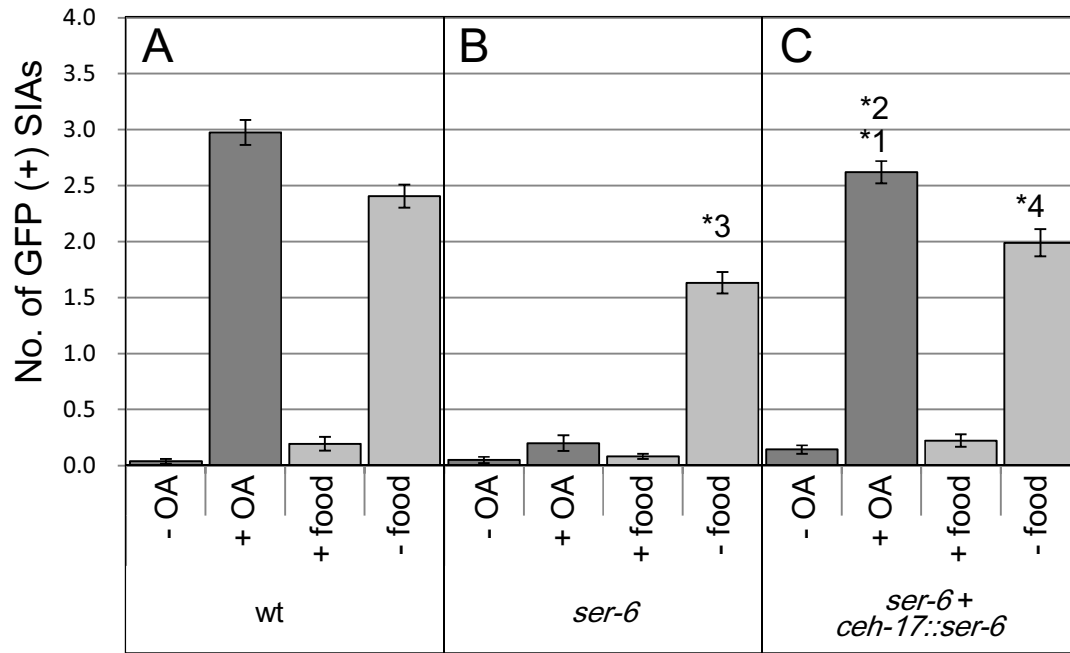
Fig.7　SIA-neuron-specific rescue of the *ser-6* CREB activation phenotype.

The transgenes *ceh-17::ser-6* and *lin-44::gfp* were introduced into a *ser-6(tm2104)* mutant carrying *cre::gfp*. The *ceh-17* promoter induces gene expression in only the SIA and ALA neurons. The *lin-44::gfp* construct was used as a co-transformation marker. Transformants were incubated on plates containing 0 or 3 mg/mL octopamine for 4 hours, or on NGM plates with or without food for 6 hours (C). At least 72 animals were tested. Error bars indicate the standard errors of the mean. CREB activity in wild-type animals and *ser-6(tm2104)* mutants shown in Fig.3E and 3G are reprinted (A, B). *1:$P<0.001$ (Tukey–Kramer multiple comparison test), compared with +OA of *ser-6* mutants. *2:$P>0.05$, compared with +OA of wild-type animals. *3:$P<0.001$, compared with -food of wild-type animals. *4:$P>0.05$, compared with -food of wild-type animals.
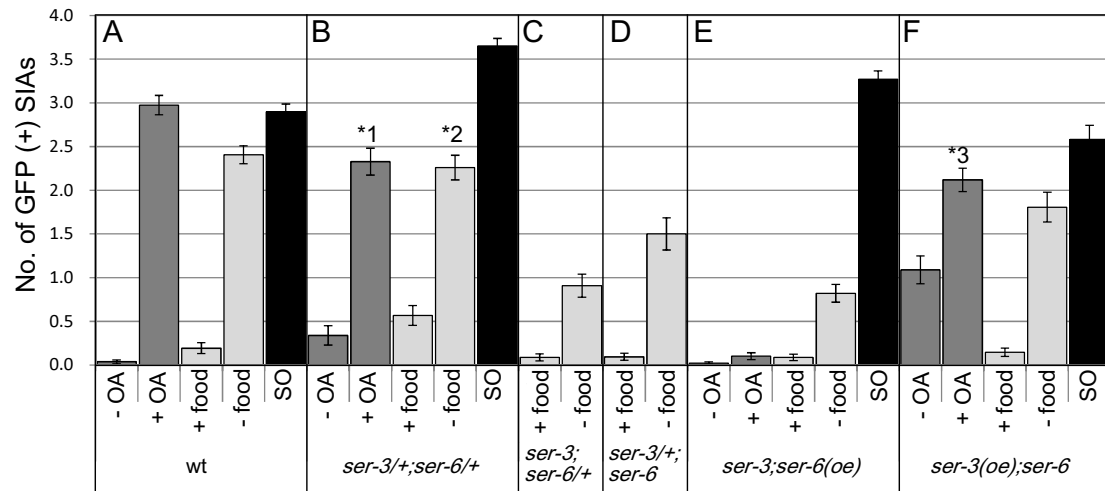
Fig.8 Octopamine- and food deprivation-mediated CREB activity in heterozygous and overexpressing animals.

Double heterozygous animals (B) , *ser-3/ser-3; ser-6/+* animals (C), *ser-3/+; ser-6/ser-6* animals (D), *ser-6*–overexpressing animals (E), and *ser-3*–overexpressing animals (F) carrying *cre::gfp* were incubated on plates containing 0 or 3 mg/mL octopamine for 4 hours, on NGM plates with or without food for 6 hours, or soaked in water (SO) in the presence of food for 4 hours. The number of GFP-expressing SIA neurons per animal was then determined. At least 43 animals were tested. Error bars indicate the standard errors of the mean. CREB activity in wild-type animals shown in Fig.3E is reprinted (A). *1:$P<0.01$ (Tukey–Kramer multiple comparison test), compared with +OA of wild-type animals. *2:$P>0.05$, compared with –food of wild-type animals. *3:$P<0.001$, compared with -OA of *ser-3(oe);ser-6* animals.
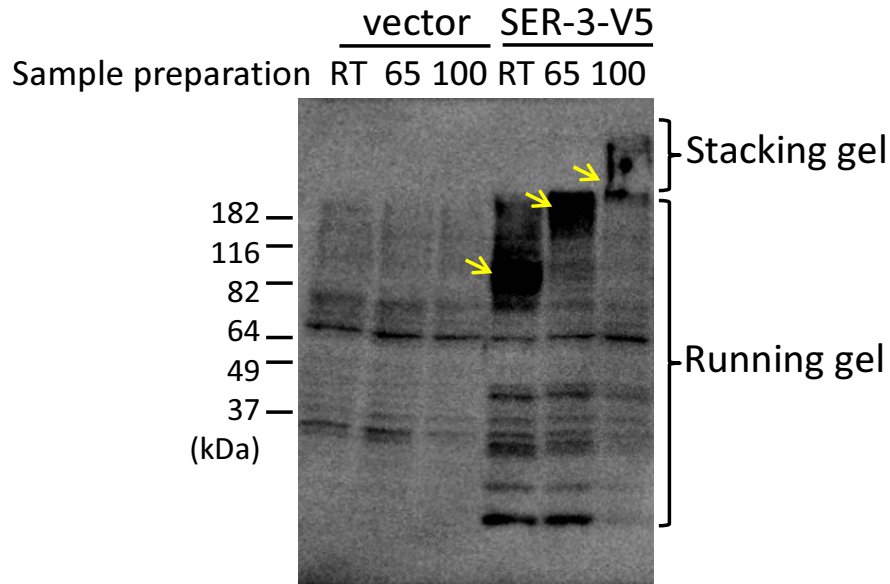
Fig.9 Western blotting of V5-tagged SER-3.

The empty vector as a negative control or the expression vector of V5-tagged *ser-3* cDNA was transfected into HEK 293 cells. After incubation for two days, the membrane of transfected cells was subjected to SDS-PAGE using a 10% acrylamide gel and Western blotting was performed with anti-V5 antibody. Samples were incubated with 1x sample buffer [0.25 M Tris-HCl (pH 6.8), 12% 2-mercaptoethanol, 4% SDS, 20% glycerol, 0.02% BPB and 8 M urea] at room temperature (RT) for 30min, at 65°C for 10 min (65) or at 100°C for 5 min (100). Arrows indicate V5-tagged SER-3. SER-3 was not detected at predicted 66 kDa, likely due to aggregation as it is common for hydrophobic proteins to aggregate when incubated at 100°C. SER-3 was detected at lower position of membrane when incubated at low temperature.
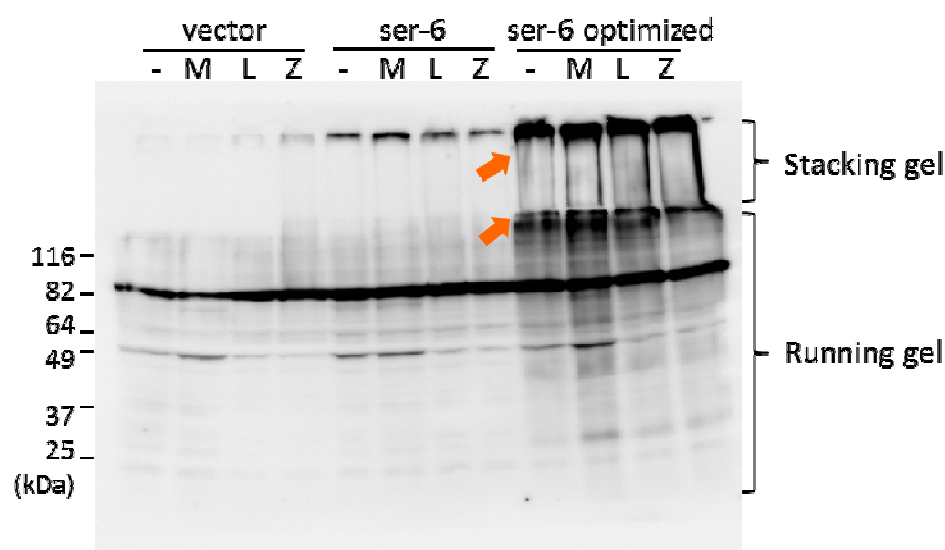
Fig.10　Western blotting of HA-tagged SER-6.

The empty vector as a negative control, expression vector of HA-tagged *ser-6* cDNA or codon-optimized *ser-6* cDNA was transfected into HEK 293 cells. After incubation for two days, the membrane of transfected cells were subjected to SDS-PAGE using a 10% acrylamide gel and Western blotting was performed with anti-HA antibody. Samples were incubated with 1x sample buffer [0.25 M Tris-HCl (pH 6.8), 12% 2-mercaptoethanol, 4% SDS, 20% glycerol, 0.02% BPB and 8 M urea] at 100°C for 5 min. SER-6 was barely detected in the presence or absence of 10^{-5}M MG-132, 10^{-8}M chlorpromazine or 10^{-8}M clozapine. Codons of *ser-6* expression vector were then optimized to human codon usage and SER-6 was detected mainly in stacking gel. Arrows indicate HA-tagged SER-6. SER-6 was not detected at predicted 44 kDa, likely due to aggregation as it is common for hydrophobic proteins to aggregate when incubated at 100°C. M: MG-132, L: chlorpromazine, Z: clozapine. Arrows indicate SER-6.

```
optimized   1' ATGATCTTGC TGTCATTGAC CCTTGGGGTG GTGAACTGCT TCGTAGTGAT CGGGAACCTT
               ***** **** * *** **** ** ** ** ** ***** * * ** ***** *** ** *
  original   1" ATGATTTTGC TATCACTGAC GCTGGGCGTC GTCAACTGTT TTGTGGTGAT CGGTAATTTG

           61' TTGGTGTTCT ACGTGATTCT GACTAAGAAG AGCCTGCAGA CAAGCACGAA TCATCTTGTT
               **** **** ********** ** ** ** *** ** * ** ***** ** * ***
           61" CTGGTTTTCT ACGTGATTCT TACCAAAAAA TCCCTTCAAA CATCAACGAA CCACTTGGTT

          121' CTGTCACTGA CAATTTCCGA TCTTCTGCTG GGTATCCTTA TTCTGCCTTT CGCCATCATC
               ********* ***** ** *** ** ** ** ******* **** ** ** ** ** **
          121" TTGTCACTGA CAATTAGTGA TCTACTTCTT GGAATCCTTA TTCTTCCATT TGCTATTATT

          181' CAGGAGCACT CTAATGAGTG GATATTTGGC CATTTTGGCT GTCGACTGTG GCTCTCAGTG
               ***** **** * ***** ** *** ** ** ** ***** * * **** ** ****** **
          181" CAGGAACACT CAAATGAATG GATTTTCGGT CACTTTGGAT GCAGACTCTG GCTCTCCGTA

          241' GACGTGTTTC TCAGCACTGC CAGCATCTAC AATCTCTTGG CGATCTCTTT CGATCGTTAC
               ** ** ** * * ***** ** ** ****** * * * ** ** ** ** ** **
          241" GATGTCTTCC TATCAACTGC ATCGATTTAT AATCTCCTTG CAATTTCATT TGACCGATAT

          301' ATGGCAGTGC GGCAACCAAT CAAATATCCC ATCATTTCCA GTACTAAGGT TGTCAGACTG
               ***** ** * * ** ***** ****** ** ***** *** ** ** ** *** ****
          301" ATGGCTGTCC GTCAGCCAAT CAAATACCCA ATCATCTCCT CCACCAAAGT GGTCCGACTA

          361' ATGACCTTTC TGGTGTGGTT CTGCTCTCTG CTGCTGGCTG TGATCCTGTT TGTTCTCCTG
               ***** ** * * ** ***** ****** ** ** ******* * ** ** ** ** *****
          361" ATGACGTTCC TCGTCTGGTT CTGCTCCCTA CTTCTGGCTG TCATACTCTT CGTGCTCCTC

          421' ACACTGAATG CGCATGACTC AGAGCCCACC AAAGACTGTC AGCCAACAAG TCTGCCTAGC
               ** ** **** * ******** ***** ** ***** **** * ** *** *** **
          421" ACCCTAAATG CTCATGACTC GGAGCCAACG AAAGATTGTC AACCCACATC TCTCCCGTCA

          481' ATGTACATCA TATTCTCTGC TATGGCGAGT TTCATAGTGC CTGCATTTGT GATGGTCATC
               ***** **** **** ** ** ***** ***** **** ********** ****** **
          481" ATGTATATCA TATTTTCGGC GATGGCCTCG TTCATTGTGC CTGCATTTGT GATGGTTATA

          541' CTGAATGTTC GCATTTTCCA GACCGTCCTG CATACTTCCA GAACCATGAC TGTCAAATCC
               ******** * * ** ** ** ** ***** ***** ** * * ** ***** ** ** **
          541" CTGAATGTCA GAATATTTCA AACGGTCCTA CATACGTCGA GGACTATGAC CGTTAAGTCG

          601' AAGAATGGCT CTCTGCGTGT CCATCGGAGA AAGGAACCTA TTATTCCCGT TAAGAAGCAC
               ********** * ** ** ** ** **** ******** * ******* ** *** ** ***
          601" AAGAATGGCT CGTTGAGGGT GCACAGGAGG AAGGAACCGA TTATTCCTGT TAAAAAACAC

          661' GACAAATACG AGACTAGGCT TTCCCACGAA GAAGAATGCG TAGGATCTCC GTCCAAAGAG
               ** ******* **** * * ******* ********** * ** ***** ** *****
          661" GATAAATACG AGACCCGTTT GAGCCACGAA GAAGAATGCG TGGGCTCTCC ATCAAAAGAA

          721' GTCATTGACC CGATACCAGT GGTAGCTGTG GTCGAGAAAC ACCACAAAAG TAGTGCCGTA
               ***** **** **** ** ** ****** ** ***** **** ***** ** **
          721" GTCATCGACC CGATTCCGGT GGTAGCAGTC GTCGAAAAC ATCACAAAG CTCGGCAGTG

          781' GATGCACCAG CCATTCGCAG CTTTCTGACA CACACAGTGG TGTTTGGGGT GTTGGAAGCC
               ******** * * ** *** ***** ** ***** **** * ***** ** *********
          781" GATGCACCCG CGATCCGCTC ATTTCTCACG CACACTGTGG TTTTTGGAGT TTTGGAAGCC

          841' AAGAAAACGA ACATCATCAA CCACATTACC CAGAAGAAGT GTATGAGGAG GAGCCTCAGA
               ** ** ** * ********** ***** ** ** ** ** * ***** * * ** **
          841" AAAAAGACCA ACATCATCAA TCACATCACA CAAAAAAAAT GTATGCGACG CTCTCTTCGA

          901' ACCGAGATTA GGGTCGCACG AACAACGGGA ATAGTGGTTG CAGCTTTCAT CGTCTGTTGG
               ** ** ** * ** ** ** ****** *** ** ** ** * * ** ***** ***** ***
          901" ACGGAAATCC GAGTAGCTCG AACAACCGGA ATCGTCGTAG CGGCCTTCAT TGTCTGCTGG

          961' ATCCCTTTCA CCACCATCTA CGTGCTGCAA GCCTATGCCG TATGCACAGT CGCTGCTGGA
               ***** ** * * ** ** ** ** ** *** ******** * **** ***** ** ** **
          961" ATCCCATTTA CAACAATATA TGTACTCCAA GCCTATGCAG TATGTACAGT TGCCGCCGGG
```

```
1021'  TGCATTCCAG  CCTCCCTCTT  TACTACCGCC  TTCTGGCTCG  GTTATAGCAA  CTCAGCCGTT
       **********  *  ** **  **    **  **  **   **  *****  *  *  **    ***    *****  **
1021"  TGCATTCCAG  CTTCACTTTT  CACGACAGCT  TTTTGGCTGG  GCTACTCCAA  TTCAGCAGTC

1081'  AACCCCATTT  TGTATGCCGC  TTTTAGCCGC  GATTTTCGGA  TAGCACTCAA  ACGGCTGTTC
       **  **  ***    *  **  **  **    **          **    **  *****  *  *  **  *****        *    *  ***
1081"  AATCCAATTC  TCTACGCAGC  GTTCTCTCGA  GACTTTCGAA  TTGCTCTCAA  GAGATTATTC

1141'  TTCCAGAAGC  CCAAGTTTGG  C
       **  *******  *  **  ***  *
1141"  TTTCAGAAGC  CAAAATTTTG  A
```

Fig.11   Sequence of optimized *ser-6.*

The upper sequence indicates *ser-6* which was optimized for human codon usage. The lower indicates the original sequence of *ser-6*. Identical nucleotides are marked with asterisks.

A

B

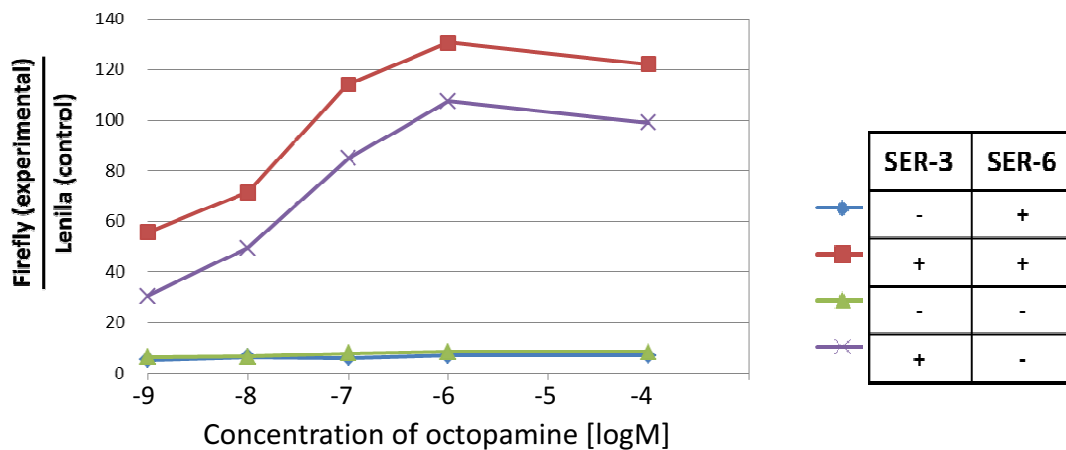|	SER-3	SER-6
(blue diamond)	-	+
(red square)	+	+
(green triangle)	-	-
(purple x)	+	-
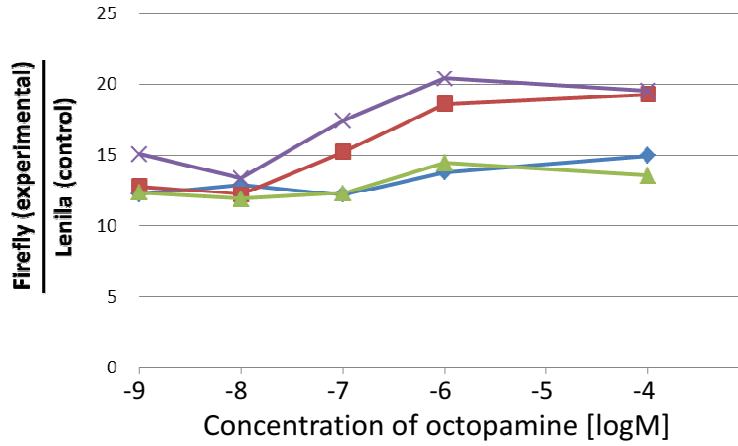
Fig.12 Octopamine induced-intracellular signaling transduced by SER-3 and SER-6 in HEK 293 cells.

The expression vectors of V5-tagged SER-3, HA-tagged SER-6 (optimized to human codon usage) or both of them, AP1-Luc (A) or CRE-Luc (B) and the renilla luciferase gene were transfected to HEK 293 cells, and exogenous octopamine (10^{-9}-10^{-4}M) was added to medium. Three hours later, luciferase activities in each condition were determined.
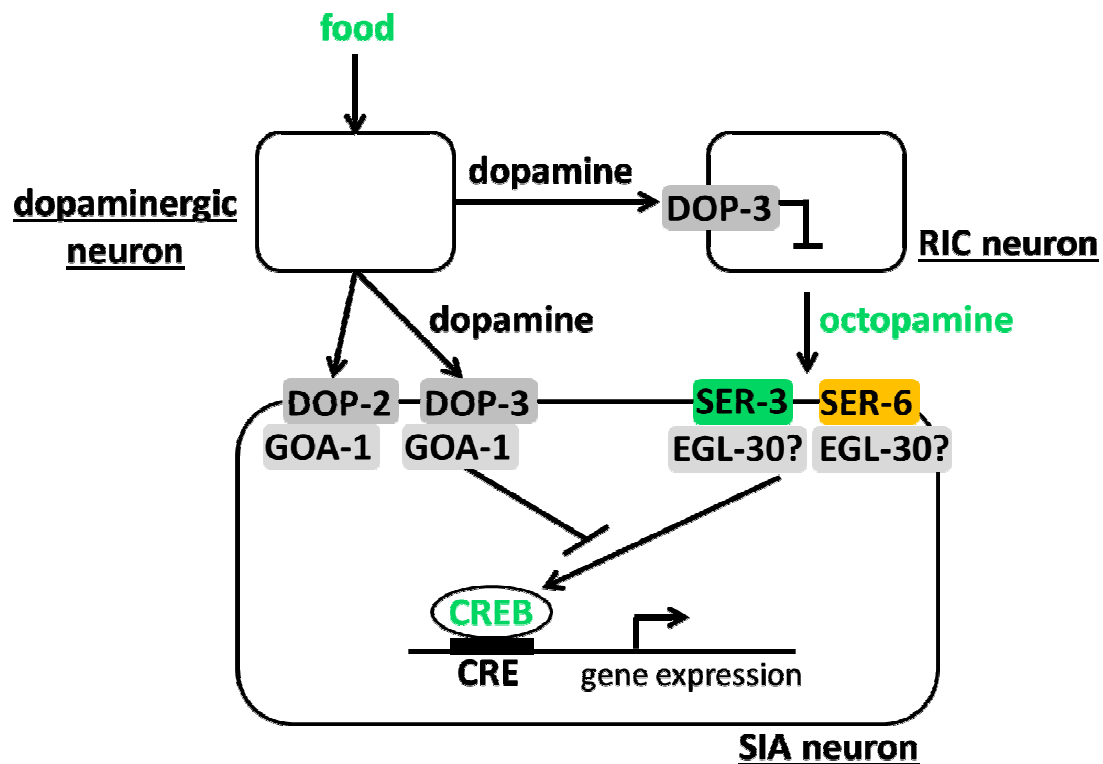
Fig.13   Function of SER-6 in food deprivation-mediated CREB activation in SIA neurons.

In this study, it was revealed that in the absence of food octopamine, which is released from the octopaminergic RIC neurons, signals through not only SER-3 but also SER-6 expressed in the SIA neurons. Loss of either SER-3 or SER-6 leads to diminished signaling, indicating that both receptors are required for normal signaling. These two similar octopamine receptors are therefore working in the same cells, and function in a non-redundant manner *in vivo*.

Fig. 2-3 and Fig. 6-8 and their legends are the pre-peer reviewed version of the following article: Yoshida M, Oami E, Wang M, Ishiura S, Suo S. 2014. Nonredundant function of two highly homologous octopamine receptors in food-deprivation-mediated signaling in *Caenorhabditis elegans*. J Neurosci Res., which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1002/jnr.23345/full].

Gene	Use	Primer sequence
ser-3	genotyping	Fw: 5'-GTTGTATGTTTTGTTGTGCTCTCT-3'
		Rv: 5'-CTCTAACTGCTCCGCCTCAA-3'
	constructing expression vector	Fw: 5'-CAAAAAAAGCTTATGGAATGGATGAG-3'
		Rv: 5'-AAATAAGGGCCCTCCGTTGGTTGGAGTGTT-3'
ser-6	reverse transcription	5'-TACATACAATTGAATTTCAG-3'
	cloning	SL1: 5'-GGTTTAATTACCCAAGTTTGAG-3'
		ser-6 Rv: 5'-GAACAATTATTACTGAACTGC-3'
	identifying expression pattern	Y54fusionA: 5'-GTTAAGCTCCTCGAACTTTCGG-3'
		Y54fusionB:5'-AGTCGACCTGCAGGCATGCAAGCTGCCCAGCGTCAGTGATAGC-3'
		Y54fusionE: 5'-CTCTCAAACTTTCCGGCGC-3'
		fusionD: 5'-AAGGGCCCGTACGGCCGACTAGTAGG-3'
		fusionF: 5'-GGAAACAGTTATGTTTGGTATATTGGG-3'
		fusionC: 5'-AGCTTGCATGCCTGCAGGTCGACT-3'
	rescue	Fw: 5'-TTCGCCACCGGTAAAAATGATTTTGCTATC-3'
		Rv: 5'-AAATAAGCGGCCGCTCAAAATTTTGGCTTC-3'
	constructing expression vector	Fw: 5'-CAAAAAAAGCTTATGATTTTGCTATC-3'
		Rv: 5'- AAATAAGTCGACTCCAAATTTTGGCTTCTG-3'
	genotyping	Fw: 5'-GTGCAGGCATGTAGGTATCT-3'
		Rv: 5'-TGACCGAGTTACGGCCTGTT-3'
tbh-1	genotyping	Fw: 5'-ATGAGAAGTGCCGTTGCTCT-3'
		Rv: 5' -AAGCAGGATCAGGAGCACAT-3'
cat-2	genotyping	Fw: 5'-GAGACATCTGAGCTAGCAGTGG-3'
		Rv: 5'-GCGAATGACGTCACTCCTATCG-3'
tyra-3	genotyping	Fw: 5'-CTCACTTCTCTAGCCACAGC-3'
		Rv: 5'-ACAGGAAGAACTCAGAAGTG-3'
octr-1	genotyping	Fw: 5'-AGTCGCTTGTCTGAATCTGG- 3'
		Rv: 5'-GAGAAGAACATGGCATGAATC- 3'
gfp	genotyping	Fw: 5'-CACTGGAGTTGTCCCAAATTCTTG- 3'
		Rv: 5'-GCCATGTGTAATCCCAGCAGC- 3'

Table 1   Primer sequences used in this study.

Fw: forward primer, Rv: reverse primer

Acknowledgments

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References

Alkema MJ, Hunter-Ensor M, Ringstad N, Horvitz HR. 2005. Tyramine functions independently of octopamine in the *Caenorhabditis elegans* nervous system. Neuron 46:247-260.

Blaxter M, Liu L. 1996. Nematode spliced leaders--ubiquity, evolution and utility. Int J Parasitol 26:1025-1033.

Blumenthal T. 2005. Trans-splicing and operons. Wormbook: 1-9.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics 77:71-94.

Carre-Pierrat M, Baillie D, Johnsen R, Hyde R, Hart A, Granger L, Segalat L. 2006. Characterization of the *Caenorhabditis elegans* G protein-coupled serotonin receptors. Invert Neurosci 6:189–205.

C. elegans sequencing consortium. 1998. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science 282:2012-2018.

Chalecka-Franaszek E, Chen H, Chuang DM. 1999. 5-Hydroxytryptamine2A receptor stimulation induces activator protein-1 and cAMP-responsive element binding with cyclic AMP-responsive element-binding protein and Jun D as common components in cerebellar neurons. Neuroscience 88:885–898.

Chase DL, Koelle MR. 2007. Biogenic amine neurotransmitters in *C. elegans*. WormBook: 1-15.

Cheng Z, Garvin D, Paguio A, Stecha P, Wood K, Fan F. 2010. Luciferase reporter assay system for deciphering GPCR pathways. Curr Chem Genomics 4:84-91.

Fire A, Albertson D, Harrison SW, Moerman DG. 1991. Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle.

Development 113:503-514.

Grohmann L, Blenau W, Erber J, Ebert PR, Strunker T, Baumann A. 2003. Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. J Neurochem 86:725-735.

Gupta A, Mulder J, Gomes I, Rozenfeld R, Bushlin I, Ong E, Lim M, Maillet E, Junek M, Cahill CM, Harkany T, Devi LA. 2010. Increased abundance of opioid receptor heteromers after chronic morphine administration. Science signaling 3:ra54.

Hague C, Lee SE, Chen Z, Prinster SC, Hall RA, Minneman KP. 2006. Heterodimers of α_{1b}- and α_{1d}-adrenergic receptors form a single functional entity. Mol Pharmacol 69:45-55.

Hague C, Uberti MA, Chen Z, Hall RA, Minneman KP. 2004. Cell surface expression of α_{1d}-adrenergic receptors is controlled by heterodimerization with α_{1b}-adrenergic receptors. J Biol Chem 279:15541-15549.

Han KA, Millar NS, Davis RL. 1998. A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. J Neurosci 18:3650-3658.

He SQ, Zhang ZN, Guan JS, Liu HR, Zhao B, Wang HB, Li Q, Yang H, Luo J, Li ZY, Wang Q, Lu YJ, Bao L, Zhang X. 2011. Facilitation of μ-opioid receptor activity by preventing δ-opioid receptor-mediated codegradation. Neuron 69:120-131.

Hobert O. 2002. PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic *C. elegans*. BioTechniques 32:728-730.

Horvitz HR, Chalfie M, Trent C, Sulston JE, Evans PD. 1982. Serotonin and octopamine in the nematode *Caenorhabditis elegans*. Science 216:1012–1014.

Huang J, Hamasaki T, Ozoe Y. 2010. Pharmacological characterization of a *Bombyx*

mori α-adrenergic-like octopamine receptor stably expressed in a mammalian cell line. Arch Insect Biochem Physiol 73:74-86.

Johannessen M, Delghandi MP, Moens U. 2004. What turns CREB on? Cell Signal 16:1211-1227.

Kimura Y, Corcoran EE, Eto K, Gengyo-Ando K, Muramatsu MA, Kobayashi R, Freedman JH, Mitani S, Hagiwara M, Means AR, Tokumitsu H. 2002. A CaMK cascade activates CRE-mediated transcription in neurons of *Caenorhabditis elegans*. EMBO Rep 3:962-966.

Kramer JM, French RP, Park EC, Johnson JJ. 1990. The *Caenorhabditis elegans rol-6* gene, which interacts with the *sqt-1* collagen gene to determine organismal morphology, encodes a collagen. Mol Cell Biol 10:2081-2089.

Lin RZ, Chen J, Hu ZW, Hoffman BB . 1998. Phosphorylation of the cAMP response element-binding protein and activation of transcription by α_1 adrenergic receptors. J Biol Chem 273:30033–30038.

Lonze BE, Ginty DD. 2002. Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605-623.

Mayr B, Montminy M. 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599-609.

Mello CC, Kramer JM, Stinchcomb D, Ambros V. 1991. Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J 10:3959-3970.

Mills H, Wragg R, Hapiak V, Castelletto M, Zahratka J, Harris G, Summers P, Korchnak A, Law W, Bamber B, Komuniecki R. 2012. Monoamines and neuropeptides interact to inhibit aversive behaviour in *Caenorhabditis elegans*. EMBO J

31:667-678.

Mohri A, Kodama E, Kimura KD, Koike M, Mizuno T, Mori I. 2005. Genetic control of temperature preference in the nematode *Caenorhabditis elegans*. Genetics. 169:1437-1450.

Murakami M, Koga M, Ohshima Y. 2001. DAF-7/TGF-β expression required for the normal larval development in *C. elegans* is controlled by a presumed guanylyl cyclase DAF-11. Mech Dev 109:27-35.

Noble T, Stieglitz J, Srinivasan S. 2013. An integrated serotonin and octopamine neuronal circuit directs the release of an endocrine signal to control *C. elegans* body fat. Cell Metab 18:672-684.

Pei L, Li S, Wang M, Diwan M, Anisman H, Fletcher PJ, Nobrega JN, Liu F. 2010. Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. Nat Med 16:1393-1395.

Petrascheck M, Ye X, Buck LB. 2007. An antidepressant that extends lifespan in adult *Caenorhabditis elegans*. Nature 450:553-556.

Pujol N, Torregrossa P, Ewbank JJ, Brunet JF. 2000. The homeodomain protein CePHOX2/CEH-17 controls antero-posterior axonal growth in *C. elegans*. Development 127:3361-3371.

Roeder T. 1999. Octopamine in invertebrates. Prog Neurobiol 59:533-561.

Sasakura H, Mori I. 2013. Behavioral plasticity, learning, and memory in *C. elegans*. Curr Opin Neurobiol 23:92-99.

Small KM, Schwarb MR, Glinka C, Theiss CT, Brown KM, Seman CA, Liggett SB. 2006. α_{2A}- and α_{2C}-adrenergic receptors form homo- and heterodimers: the heterodimeric state impairs agonist-promoted GRK phosphorylation and

β-arrestin recruitment. Biochemistry 45:4760-4767.

Srinivasan S, Sadegh L, Elle IC, Christensen AG, Faergeman NJ, Ashrafi K. 2008. Serotonin regulates *C. elegans* fat and feeding through independent molecular mechanisms. Cell Metab 7:533-544.

Stanasila L, Perez JB, Vogel H, Cotecchia S. 2003. Oligomerization of the α_{1a}- and α_{1b}-adrenergic receptor subtypes. Potential implications in receptor internalization. J Biol Chem 278:40239-40251.

Sulston J, Dew M, Brenner S. 1975. Dopaminergic neurons in the nematode *Caenorhabditis elegans*. J Comp Neurol 163:215-226.

Suo S, Culotti JG, Van Tol HH. 2009. Dopamine counteracts octopamine signalling in a neural circuit mediating food response in *C. elegans*. EMBO J 28:2437-2448.

Suo S, Ishiura S. 2013. Dopamine modulates acetylcholine release via octopamine and CREB signaling in *Caenorhabditis elegans*. PLoS One 8:e72578.

Suo S, Kimura Y, Van Tol HH. 2006. Starvation induces cAMP response element-binding protein-dependent gene expression through octopamine-Gq signaling in *Caenorhabditis elegans*. J Neurosci 26:10082-10090.

Thonberg H, Fredriksson JM, Nedergaard J, Cannon B. 2002. A novel pathway for adrenergic stimulation of cAMP-response-element-binding protein (CREB) phosphorylation: mediation via α_1-adrenoceptors and protein kinase C activation. Biochem J 364:73–79.

White JG, Southgate E, Thomson JN, Brenner S. 1986. The structure of the nervous system of the nematode *Caenorhabditis elegans*. Philos Trans R Soc Lond B Biol Sci 314:1-340.

Wood WB. 1988. The Nematode *Caenorhabditis elegans*. Cold Spring Harbor

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Wragg RT, Hapiak V, Miller SB, Harris GP, Gray J, Komuniecki PR, Komuniecki RW. 2007. Tyramine and octopamine independently inhibit serotonin-stimulated aversive behaviors in *Caenorhabditis elegans* through two novel amine receptors. J Neurosci 27:13402-13412.

Zhu WZ, Chakir K, Zhang S, Yang D, Lavoie C, Bouvier M, Hébert TE, Lakatta EG, Cheng H, Xiao RP. 2005. Heterodimerization of β_1- and β_2-adrenergic receptor subtypes optimizes β-adrenergic modulation of cardiac contractility. Circ Res 97:244-251.