Maintenance of neuronal positions in organized ganglia by SAX-7, a *Caenorhabditis elegans* homologue of L1

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The L1 family of cell adhesion molecules is predominantly expressed in the nervous system. Mutations in human L1 cause neuronal diseases such as HSAS, MASA, and SPG1. Here we show that *sax-7* gene encodes an L1 homologue in *Caenorhabditis elegans*. In *sax-7* mutants, the organization of ganglia and positioning of neurons are abnormal in the adult stage, but these abnormalities are not observed in early larval stage. Misplacement of neurons in *sax-7* mutants is triggered by mechanical force linked to body movement. Short and long forms of SAX-7 exhibited strong and weak homophilic adhesion activities in *in vitro* aggregation assay, respectively, which correlated with their different activities *in vivo*. SAX-7 was localized on plasma membranes of neurons *in vivo*. Expression of SAX-7 only in a single neuron in *sax-7* mutants cell-autonomously restored its normal neuronal position. Expression of SAX-7 in two different head neurons in *sax-7* mutants led to the forced attachment of these neurons. We propose that both homophilic and heterophilic interactions of SAX-7 are essential for maintenance of neuronal positions in organized ganglia.

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Introduction

The nervous system is an elaborate three-dimensional structure, in which the positions of neurons are highly organized. How ganglia are organized and how organized ganglia are maintained are important questions in developmental neurobiology. The nematode *Caenorhabditis elegans* is a useful organism in which to address these questions. The morphol-

ogy and connections of 302 neurons that make up the nervous system are thoroughly described (Brenner, 1974; White *et al.*, 1976; Sulston and White, 1980; White *et al.*, 1986; Durbin, 1987). Approximately half of all neuronal cell bodies reside near the nerve ring where synaptic connections between many neurons are made. Although many cell bodies are concentrated near the nerve ring, the positions of individual cell bodies are constant among different animals and are maintained at the same place throughout their life (Ward *et al.*, 1975; Ware *et al.*, 1975; Sulston and White, 1980; White *et al.*, 1986).

The L1 family of neural cell adhesion molecules belongs to the immunoglobulin superfamily and features six immunoglobulin-like domains, five fibronectin type III repeats, a transmembrane domain, and a cytoplasmic tail. Several members of the L1 family have been characterized: in vertebrates, L1CAM/NgCAM, NrCAM/BRABO, CHL1, and neurofascin; and in invertebrates, neuroglian (Moos *et al.*, 1988; Grumet, 1991; Hortsch, 1996, 2000; Brummendorf *et al.*, 1998; Rougon and Hobert, 2003). These molecules are predominantly expressed in peripheral and central nervous systems. L1 molecules have been implicated in the processes of neuronal cell migration, myelination, axonal growth, pathfinding, and fasciculation via homophilic and heterophilic interactions with other molecules (Lindner *et al.*, 1983; Grumet, 1991, 1997; Hortsch, 1996, 2000; Burden-Gulley *et al.*, 1997; Brummendorf *et al.*, 1998; Brummendorf and Lemmon, 2001; Rougon and Hobert, 2003). Mutations in the human *L1CAM* gene lead to several X-linked neuronal disorders, such as HSAS (hydrocephalus), MASA (mental retardation, aphasia, shuffling gait, adducted thumb), and SPG1 (spastic paraplegia type 1) (Rosenthal *et al.*, 1992; Van Camp *et al.*, 1993; Jouet *et al.*, 1994; Uyemura *et al.*, 1994; Vits *et al.*, 1994; Wong *et al.*, 1995; Kamiguchi *et al.*, 1998; Kenwrick and Doherty, 1998). The phenotypes of L1CAM-deficient mouse are pleiotropic. Mutant mice exhibit small body size, delayed motor responses, weakness of their hind limbs, abnormal corticospinal tracts, and enlarged lateral ventricles (Dahme *et al.*, 1997; Cohen *et al.*, 1998; Demyanenko *et al.*, 1999). Hippocampal mossy fiber and olfactory axons are affected in CHL1-deficient mice (Montag-Sallaz *et al.*, 2002). In *Drosophila*, mutations in the *neuroglian* gene lead to lethality and affect motor neuron pathfinding (Bieber *et al.*, 1989; Hall and Bieber, 1997). Since mutations of other genes of L1 family have not yet been identified, little is known about *in vivo* functions of many L1 members.

In this study, we showed that *sax-7* encodes a *C. elegans* homologue of L1 and that SAX-7 is required for maintenance of neuronal positions. The expression of SAX-7 only in a single neuron of *sax-7* mutants cell-autonomously restored neuronal positions, suggesting that SAX-7 act as a heterophilic cell adhesion molecule. Expression of SAX-7 in two different head neurons in *sax-7* mutants led to the forced

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attachment of these neurons. Our results led us to propose that SAX-7, a *C. elegans* homologue of L1, regulates organization of ganglia and maintenance of neuronal positions via both homophilic and heterophilic adhesion.

Results

sax-7 encodes a *C. elegans* homologue of L1

In *C. elegans*, environmental temperature is sensed by AFD thermosensory neurons and thermal information is transduced to AIY interneurons (Mori and Ohshima, 1995). The cell bodies of AFD are located posterior to the nerve ring, the

dendrites extend to the tip of the head where environmental stimuli are received, and the axons project to the nerve ring (Perkins *et al*, 1986; White *et al*, 1986) (Figure 1A). The cell bodies of AIY interneurons are located posterior to the nerve ring and the axons project to nerve ring where synaptic connections to AFD and other neurons are formed (White *et al*, 1986) (Figure 1A). We conducted a visual screen for genes required for development and maintenance of AFD and AIY by introducing AFD- and AIY-specific GFP markers into the *mut-7* strain, in which transposon insertions occur frequently (Chalfie *et al*, 1994; Hobert *et al*, 1997; Ketting *et al*, 1999). We isolated *nj13* and *nj52* mutants that show abnormal

Figure 1 Disrupted cell body positions of *sax-7* mutants. (A–J) Phenotypes of cell body positions in adult stage. (A, B) Neuronal morphology of AFD thermosensory neurons and AIY interneurons: (A) wild type and (B) *sax-7* mutants. Upper panels are merged DIC and GFP images. Lower panels are GFP images. The cell body of AFD is indicated by a white arrow. The cell body of AIY is indicated by a yellow arrow. The position of the nerve ring is indicated by an arrowhead. (C, D) Neuronal morphology of AWB and AWC olfactory neurons: (C) wild type and (D) *sax-7* mutants. The cell body of AWB is indicated by a white arrow. The cell body of AWC is indicated by a yellow arrow. (E, F) Neuronal morphology of RIA interneurons: (E) wild type and (F) *sax-7* mutants. The cell body of RIA is indicated by a white arrow. (G, H) Sensory neurons in head regions: (G) wild type and (H) *sax-7* mutants. Upper panels are merged DIC and GFP images. The position of the majority of sensory neurons is indicated by a white arrow. (I, J) Several interneurons in head regions: (I) wild type and (J) *sax-7* mutants. The position of the majority of interneurons is indicated by a white arrow. Anterior is left and dorsal is up for all animals. Scale bar is 20 μ m.

neuronal positioning. AFD cell bodies were mislocalized anterior to the nerve ring in almost all of *nj13* and *nj52* animals (Figure 1A and B, and Table I). AIY cell body positions were also abnormal in *nj13* and *nj52* mutants. Although AIY cell bodies are localized on the ventral side in wild type, they were often mislocalized anteriorly, posteriorly, or on the dorsal side in *nj13* and *nj52* mutants (Figure 1A and B, and Supplementary data 1). The neuronal phenotype of *nj13* and *nj52* mutants was indistinguishable from the phenotype of *sax-7(ky146)* mutants, whose sensory axons are misplaced relative to their cell bodies (Zallen *et al*, 1999). We found that *nj13* and *nj52* are allelic to *sax-7(ky146)* mutation.

Cloning by transposon display (Korswagen *et al*, 1996; Wicks *et al*, 2000) revealed that *sax-7* encodes a homologue of neural cell adhesion molecule L1, which is a member of the immunoglobulin superfamily and was previously designated as LAD-1 (Teichmann and Chothia, 2000; Chen *et al*, 2001; Vogel *et al*, 2003). The cosmid clone C18F3 including only a single ORF for this *C. elegans* homologue of L1 rescued the abnormal neuronal positions of both AFD and AIY neurons (Table I and Supplementary data 2). In *sax-7(nj13)* mutants, the transposon Tc4 was inserted between the second and third Fibronectin type III (FNIII) domain, which likely results in the early appearance of a stop codon. Similarly, both *sax-7(ky146)* and *sax-7(nj52)* mutations likely result in the early appearance of stop codons (Figure 2A and B, and Supplementary data 2). Characterization of *sax-7* cDNA clones revealed that there are two forms of *sax-7* cDNA, long and short forms (The cDNA sequences of *C. elegans sax-7* long and *sax-7* short were deposited in DDBJ with accession numbers AB206669 and AB206670). Six extracellular Ig-like domains and five extracellular FNIII repeats are characteristic of L1 family neuronal adhesion molecules, which is consistent with the predicted structure of the long form of SAX-7. The short form lacks the first two Ig-like domains of the long form (Figure 2B). The SAX-7 long form has 27 % identity and

64 % similarity to *Drosophila* neuroglian, 26 % identity and 58 % similarity to human NrCAM, and 23 % identity and 58 % similarity to human L1CAM (Figure 2D).

Positions of neurons and organization of ganglia are defective in *sax-7* mutants

sax-7(ky146) was previously reported to affect the localization of seven pairs of sensory neurons in the amphid (Zallen *et al*, 1999). We labeled two sensory neurons AWB and AWC of *sax-7* mutants with *odr-1::GFP* and observed their positions in more detail (L'Etoile and Bargmann, 2000). In wild type, cell bodies of AWB and AWC are positioned in the neighborhood of the cell body of AFD. AWB is localized dorso-laterally and AWC was localized ventro-laterally to AFD (Sulston and White, 1980; White *et al*, 1986) (Figures 1A, C and 7A). The abnormality of AWB positions was similar to the abnormality of AFD in *sax-7* mutants. The abnormality of AWC positions was somewhat milder than the abnormality of AFD in *sax-7* mutants (Figure 7D). The quantitative evaluations of abnormalities of AFD, AWB, and AWC relative to pharynx are shown in Supplementary data 3. The RIA interneuron is located very close to the AFD sensory neuron in wild type (Sulston and White, 1980; White *et al*, 1986) (Figure 1E). RIA labeled with *glr-3::GFP* (Brockie *et al*, 2001) was positioned in a similarly abnormal location to AFD in *sax-7* mutants (Figure 1A, B, E, and F).

We labeled almost all sensory neurons and several interneurons of *sax-7* mutants with *osm-6p::GFP* and *glr-1p::GFP*, respectively (Hart *et al*, 1995; Maricq *et al*, 1995; Collet *et al*, 1998). In wild type, many sensory neurons make tightly linked ganglia that are situated posterior to the nerve ring (Ward *et al*, 1975; Ware *et al*, 1975; White *et al*, 1986) (Figure 1G). In *sax-7* mutants, the structure of sensory ganglia was disturbed, and most of the sensory neurons were mislocalized anterior to nerve ring (Figure 1H). *glr-1p::GFP*-expressing interneurons were also scattered and mislocalized

Table I Abnormality of cell body positions in *sax-7* mutants

Genotype	Normal	Partially abnormal	Abnormal	<i>n</i>
<i>Adult stage</i>				
Wild type	99%	1%	0%	190
<i>sax-7(nj13)</i>	1%	15%	84%	221
<i>sax-7(ky146)</i>	2%	11%	87%	267
<i>sax-7(nj52)</i>	1%	15%	84%	238
<i>sax-7(nj48)</i>	1%	9%	90%	227
<i>sax-7(nj13)</i> + cosmid C18F3	87%	8%	5%	354
<i>sax-7(nj48)</i> + cosmid C18F3	89%	10%	1%	291
<i>unc-54(e190)</i>	96% ^a	3%	1%	154
<i>unc-54(e190); sax-7(nj48)</i>	62% ^b	17%	21%	313
<i>First larval stage</i>				
Wild type	100%	0%	0%	37
<i>sax-7(nj13)</i>	87%	12%	1%	62
<i>sax-7(nj146)</i>	90%	9%	1%	65
<i>sax-7(nj52)</i>	89%	11%	0%	53
<i>sax-7(nj48)</i>	85%	12%	3%	115

Cell body phenotypes were scored in adult and the first larval stage with the *H13::GFP* marker, which labels a single pair of AFD thermosensory neurons. We categorized cell bodies localized posterior to nerve ring as normal, cell bodies that overlap with nerve ring when observed from lateral side as partially abnormal, and cell bodies localized anterior to nerve ring as abnormal.

n indicates number of animals observed.

^aIncluding 2 % animals whose AFD are more posterior than those of wild type.

^bIncluding 5 % animals whose AFD are more posterior than those of wild type.

Figure 2 *sax-7* encodes a *C. elegans* homologue of L1. (A) Genomic organization of the SAX-7 coding region with mutation sites. Exons are indicated by open boxes. (B) Protein structure of SAX-7 long and short forms. Long form SAX-7 has six Ig-like domains, whereas short form SAX-7 has four Ig-like domains. (C) Western blotting. Left panel: 160 kDa (long) and 140 kDa (short) proteins were detected in wild type. The 140 kDa (short) protein was detected in *nj53* N-terminal deletion mutants. No bands were detected in other *sax-7* mutants. Right panel: *sax-7(nj48)* mutants carrying cosmid clone C18F3, *unc-14p::longsax-7cDNA*, or *unc-14p::shortsax-7cDNA* showed both bands, long band, or short band, respectively. (D) Phylogenetic analysis of SAX-7 and related proteins. Full-length amino acid sequences are compared for analysis. UNC-40 is the *C. elegans* homologue of DCC that bears four Ig-like domains and six FNIII repeats (Chan *et al*, 1996). SAX-3 is the *C. elegans* homologue of ROBO that bears five Ig-like domains and three FNIII repeats (Zallen *et al*, 1998).

anterior to the nerve ring (Figure 1I and J). In wild type, the dendrites of head sensory neurons are fasciculated and their bundles run straight to the tip of head where environmental stimuli are sensed (Ward *et al*, 1975; Ware *et al*, 1975) (Figure 1G). Although not as severe as the defect in cell body position, we sometimes observed that fasciculated dendrites were loosely bundled in *sax-7* mutants (Figure 1H and data not shown). We found that tail sensory neurons were also misplaced in *sax-7* mutants (data not shown).

We examined whether neuronal abnormalities exhibited by *sax-7* mutants affect sensory behaviors of *C. elegans* (Ward, 1973; Hedgecock and Russell, 1975). Surprisingly, both thermotaxis and chemotaxis to NaCl were almost normal in *sax-7* mutants (thermotaxis: wild type: normal = 90%,

abnormal = 10% ($n = 60$); *sax-7*: normal = 85%, abnormal = 15% ($n = 60$); chemotaxis to NaCl; wild type: normal = 92%, abnormal = 8% ($n = 50$); *sax-7*: normal = 85%, abnormal = 15% ($n = 50$)). These results suggest that neuronal network formation, axonal projections, and synaptic connections are largely intact in *sax-7* mutants.

SAX-7/L1 is required for maintenance of neuronal positions, not for neuronal development

sax-7(ky146) was previously reported to have a defect in the postembryonic maintenance of sensory neuron morphology, rather than initial placement (Zallen *et al*, 1999). To evaluate these possibilities more rigorously, we traced developmental stages and scored positions of AFD and AIY. The neuronal positions of AFD and sensory neurons were normal in *sax-7* mutants in the first larval stage, whereas abnormal neuronal positioning clearly appeared after the second larval stage (Figure 3A–E and Table I) (Zallen *et al*, 1999), suggesting that neurogenesis, specification of neurons, and formation of neuronal connections are normal in *sax-7* mutants.

Misplacement of neurons in *sax-7* mutants is triggered by mechanical force linked to body movement

It is possible that the defect of neuronal positioning in *sax-7* mutants is triggered by mechanical force linked to their body movement. To test this possibility, we examined abnormal

Figure 3 SAX-7/L1 is required for maintenance of neuronal positions and not required for neuronal development. (A–D) Neuronal morphology of AFD and AIY (A, B) and head sensory neurons (C, D) in the first larval stage: (A, C) wild type and (B, D) *sax-7* mutants. Cell body positions of *sax-7* mutants are indistinguishable from those of wild type. The positions of head sensory neurons are generally normal in *sax-7* mutants in the first larval stage. White arrows indicate the cell body of AFD (A, B) or ganglia of sensory neurons (C, D) and arrowheads indicate the nerve ring. Scale bar is 20 μ m. (E) Graphs depicting scores of cell body phenotype of AFD. The X-axis indicates the developmental time. Time 0 is when eggs were laid. After cultivation at 20°C for 16, 33, 48, 72, 92, and 110 h, neuronal positions were scored (for each time point, > 100 neurons were counted). The phenotypic pattern at 16 h corresponds to the pattern of the first larval stage shown in Table I.

neuronal positions of *sax-7* mutation in *unc-54* mutants, which show severe movement abnormality due to defective body wall muscle formation (Karn *et al*, 1983; Dibb *et al*, 1985). The abnormal AFD positions were recovered in double mutants (Table I). The suppression of AFD abnormal positions suggests that head movement causes the mispositioning of neurons and that SAX-7/L1-mediated cell adhesion system is critical for antagonizing mechanical force accompanied with movement.

Long and short form SAX-7 show different rescue activities for neuronal placement

The long form has six Ig-like domains, whereas the short form lacks the initial two Ig-like domains of the long form (Figure 2B). To examine which form of SAX-7 affects neuronal positioning, we isolated *sax-7(nj47)* and *sax-7(nj53)* mutants that lack the initial methionine and the signal sequence of the long form as well as *sax-7(nj48)* mutant that lacks the signal sequence of the short form and part of the Ig-like domain of the long form (Figure 2A and B, and Supplementary data 2). We examined the protein expression of *sax-7* alleles by Western blotting using LAD-1/SAX-7 antibody (Chen *et al*, 2001). The 160 and 140 kDa bands were detected in wild type and these two bands were not detected in *sax-7(nj48)*, *sax-7(nj13)*, *sax-7(ky146)*, and *sax-7(nj52)* mutants (Figure 2C), suggesting that these *sax-7* alleles are null. We confirmed that 160 and 140 kDa bands correspond to long and short forms, respectively. *sax-7(nj48)* null mutants expressing the genomic clone showed both 160 and 140 kDa bands, *sax-7(nj48)* mutants expressing the long form showed only 160 kDa band, and *sax-7(nj48)* mutants expressing the short form showed only 140 kDa band (Figure 2C). *sax-7(nj53)* mutants lacked long form and retained short form, although the expression level of SAX-7 short protein seemed upregulated (Figure 2C). The long form-specific deletion mutants *sax-7(nj47)* and *sax-7(nj53)* showed normal neuronal positions (Figure 4A and B, and Table II) and *sax-7(nj48)* mutants showed abnormal neuronal positioning similar to the abnormal positioning of *nj13*, *nj52*, and *ky146* mutants (Figure 4A and C, and Table II). These results suggest that the short form of SAX-7 may be sufficient for maintaining neuronal positions, whereas the long form is not essential.

The previous report postulated that the first three Ig domains of LAD-1/SAX-7 caused a dominant-negative effect, resulting in morphologically abnormal phenotype (*vab* phenotype) and uncoordinated movement (Chen *et al*, 2001). We, however, obtained no solid evidence to indicate that *sax-7* null mutations caused these defects (data not shown). The GFP-fused extracellular portion of SAX-7 used as a dominant-negative form in the previous study might have disrupted other cell adhesion molecules, thereby artificially causing severe defect in morphogenesis.

We next expressed a *sax-7* short form cDNA in nearly all neurons of *sax-7(nj48)* mutants using the pan-neuronal promoter *unc-14p* (Ogura *et al*, 1997). The expression of *sax-7* short form cDNA almost completely rescued the abnormal neuronal positioning of *sax-7(nj48)* mutants (Figure 4E and Table II). We also expressed a *sax-7* long form cDNA in nearly all neurons of *sax-7(nj48)* mutants. The SAX-7 long form showed weaker rescue activity than the short form (Figure 4C–E and Table II). The rescue activity of the

Figure 4 SAX-7 long and short forms show different adhesion activities for neuronal positioning *in vivo*. (A–C) Cell body phenotypes of different *sax-7* alleles: (A) wild type, (B) *sax-7(nj53)* long form-specific deletion mutant showing normal neuronal positions, and (C) *sax-7(nj48)* null mutant showing abnormal neuronal positions. (D, E) Different rescuing ability of long and short SAX-7. (D) In about 40% of *sax-7(nj48)* animals transgenic with *unc-14p::longcDNA*, in which SAX-7 long form is expressed in nearly all neurons, AFD positions were overlapped with the nerve ring, implicating weak adhesion activity of the long form. (E) In over 80% of *sax-7(nj48)* animals transgenic with *unc-14p::shortcDNA*, AFD positions were normal, implicating strong adhesion activity of the short form. A white arrow indicates the cell body of AFD and an arrowhead indicates the nerve ring. Scale bar is 20 μ m. See Table II for summary.

SAX-7 long form remained weak even in *sax-7(nj48)* animals transgenic with 10-fold higher concentrations of the long form cDNA (normal = 11%, partially abnormal = 38%, abnormal = 51%, $n = 264$). The difference in rescue activity between two forms coincided with the phenotypic difference between two deletion mutants: *sax-7(nj47)* and *sax-7(nj53)* retaining the SAX-7 short form were normal and *sax-7(nj48)* without both long and short forms were abnormal in neuronal positioning (Figure 4A–E and Table II).

SAX-7 is localized on the neuronal plasma membrane in cell bodies, axons, and dendrites

The previously reported antibody against SAX-7/LAD-1 recognizes the cytoplasmic tail of the protein (Chen *et al*, 2001), which likely stains both short and long forms of SAX-7. We determined the intracellular localization of SAX-7 short form using GFP-tagged SAX-7 (Figure 5A). The *sax-7shortcDNA::GFP* gene proved functional, since the expression of *sax-7shortcDNA::GFP* under the *unc-14* promoter efficiently rescued the abnormal neuronal positioning of both AFD and AIY (data not shown). When *sax-7shortcDNA::GFP* was expressed in sensory neurons or only in AFD neurons using the *osm-6* or *gcy-8* promoter, respectively, SAX-7short::GFP was localized to the plasma membranes of cell bodies, axons, and dendrites (Figure 5B–E). The fluorescence of the SAX-7short::GFP was especially enriched on the surface, where neuron-to-neuron contacts are likely to occur (Figure 5C), implicating that SAX-7 can play a role in the adhesion between neurons within sensory ganglia.

Homophilic adhesion activities of SAX-7 long and short forms in culture system

We expressed SAX-7 protein in cultured cells and quantified cell aggregation using BmN4 cells of the silkworm *Bombyx mori* larvae and silkworm baculovirus expression system (Maeda *et al*, 1985). We prepared BmN4 cells expressing SAX-7 short form, BmN4 cells expressing SAX-7 long form, and BmN4 cells infected with parental virus as control. BmN4 cells themselves did not show cell aggregation activity (Figure 6A). Immunostaining analysis revealed that both short and long form SAX-7 were expressed on the cell surface at similar level (Figure 6B and data not shown) (Chen *et al*, 2001). Short form-expressing cells made large aggregates ($N_t/N_o=0.12$), while long form-expressing cells made much smaller aggregates ($N_t/N_o=0.49$) (Figure 6C–E). We mixed long form- and short form-expressing cells. Although we apparently observed cell aggregations of long form- and short form-expressing cells (data not shown), aggregation of short form-expressing cells was too strong to determine the aggregation ratio of long-short combination (data not shown). These results suggest that the homophilic adhesion of short form has stronger adhesion activity than long-long adhesion and that both forms of SAX-7 can interact. Importantly, the homophilic adhesion activities of short and long form SAX-7 in aggregation assays correlated well with their rescue

Figure 5 SAX-7 is localized on plasma membranes, axons, and dendrites of neurons. (A) GFP-tagged *sax-7* short cDNAs were expressed in sensory neurons or AFD thermosensory neurons using *osm-6* or *gcy-8* promoter. (B, C) Localization of SAX-7::GFP in sensory neurons. (B) SAX-7::GFP is localized on plasma membranes (white arrow), axons (arrowhead), and dendrites (yellow arrow). (C) SAX-7::GFP is enriched in the region of neuron-to-neuron contacts (white arrow). (D, E) Localization of SAX-7::GFP in the single neuron AFD. (D) SAX-7::GFP is localized on the plasma membrane of AFD, axon (arrowhead), and dendrite (yellow arrow). (E) SAX-7::GFP is localized on the plasma membrane of AFD (white arrow). Wild-type animals of the second larval stage were observed for SAX-7::GFP localization. Scale bar is 20 μm.

Table II Cell adhesion activities of SAX-7 long and short forms *in vivo*

Genotype				<i>n</i>	
Wild type		99%	1%	0%	190
<i>sax-7(nj47)</i>		100%	0%	0%	100
<i>sax-7(nj53)</i>		100%	0%	0%	100
<i>sax-7(nj48)</i>		1%	9%	90%	227
<i>sax-7(nj48)</i> Ex; [<i>unc-14p::long cDNA</i>]		11%	44%	45%	617
<i>sax-7(nj48)</i> Ex; [<i>unc-14p::short cDNA</i>]		85%	12%	3%	397

Cell body phenotypes were scored in the long form-specific deletion mutants *sax-7(nj47)* and *sax-7(nj53)*, and the both form deletion mutant *sax-7(nj48)*, *sax-7(nj48)* carrying the long form SAX-7, or *sax-7(nj48)* carrying the short form SAX-7. A measure of 5 ng/μl of *unc-14p::short cDNA* or *unc-14p::long cDNA* was injected into *sax-7(nj48)* mutants and transgenic animals of more than six independent lines were observed. Molecular structure in black color indicates intact endogenous SAX-7 proteins, gray color indicates inactive state of SAX-7, and green color indicates exogenous SAX-7. *n* indicates number of animals observed. *nj47* mutants show larval arrest phenotype presumably due to the side mutation (see Materials and methods). *nj47* animals that corresponded to L3 or L4 larval stage were scored for neuronal placement phenotype.

Figure 6 Long and short form SAX-7 show different homophilic adhesive activities in culture system. (A) BmN4 cells infected with empty BmNPV virus did not form cell aggregations (control-control; C-C). Cells were grown for 4 days after virus infection and subjected to cell aggregation assays (see Materials and methods). (B) SAX-7 was localized to the cell membrane in BmN4 cells correctly. Cells were infected with BmNPV virus expressing SAX-7 long form for 4 days, fixed in formaldehyde, and incubated with anti-SAX-7 antibody before staining with Cy3 anti-rabbit secondary antibody. (C) SAX-7 long form-expressing cells aggregated via homophilic bindings (L-L). (D) BmN4 cells infected with BmNPV virus expressing SAX-7 short form made large aggregates (S-S). (E) Quantification of cell aggregation in C-C, L-L, and S-S. The ratio of total particle number after rotated incubation (N_t) was calculated after 60 min from the initial point. The data in panel E are mean $\pm$ s.e.m. from five experiments. * $P < 0.01$; ** $P < 0.001$. Scale bars are 100 μ m (A, C, and D) and 10 μ m (B).

activities for neuronal positioning *in vivo* (Figures 4D, E, and 6C-E, and Table II).

Heterophilic adhesion of SAX-7 *in vivo*

To address whether SAX-7 could interact heterophilically with other cell adhesion molecules *in vivo*, we expressed SAX-7 short form only in AFD neuron of *sax-7(nj48)* mutants and asked if the position of AFD could be restored. *gcy-8p::dsRed2* that labeled the AFD neurons, and *odr-1p::GFP* that labeled the AWB and AWC neurons were also expressed in order to monitor the neuronal positions of AFD, AWB, and AWC. The AFD positions were partially rescued in *sax-7(nj48)* transgenic animals expressing SAX-7 short form only in AFD, while the positions of AWB and AWC remained abnormal (Figure 7A-D). Only 1% of AFD cell bodies were normally localized posteriorly, 9% overlapped with the nerve ring, and 90% were mislocalized anterior to the nerve ring in *sax-7(nj48)* mutants (Figure 7D). By contrast, the fraction of animals with proper localization of AFD increased greatly among *sax-7(nj48)* animals with highly expressing SAX-7 short form only in AFD. The proportion of normally localized AFD increased from 1 to 14% and the proportion of AFD that overlapped with the nerve ring increased from 9 to 25%, while the positions of the neighboring AWB and AWC neurons were only slightly affected (Figure 7D). The strong restoration of AFD positions by high expression of SAX-7

short form could be simply the result of overexpression or some artificial effect. We then examined the dosage dependency of cell-autonomous restoration, by injecting *gcy-8p::sax-7shortcDNA* at 5, 50, 150, 300, and 500 ng/ μ l, and observed dosage-dependent restoration of AFD positions when the *gcy-8p::sax-7shortcDNA* was injected at more than 50 ng/ μ l (Figure 7E). These results suggest that the interaction of SAX-7 with a heterophilic partner contributes to the maintenance of neuronal positions.

Homophilic adhesion of SAX-7 *in vivo*

We asked if SAX-7 could interact homophilically *in vivo*. We expressed SAX-7 short form in two separate head neurons in *sax-7(nj48)* null mutants and examined whether the two neurons could adhere with each other in live animals. We expressed *sax-7shortcDNA* in AWB and AWC of *sax-7(nj48)* mutants and the attachment of AWB and AWC was scored (Figure 7F-I). The result showed that $15 \pm 5\%$ of AWB and AWC on the same side was attached in *sax-7(nj48)* mutants and $8 \pm 5\%$ was attached in wild type, whereas $30 \pm 4\%$ of these two cells was attached in the transgenic animals (Figure 7I).

The two cell attachment in transgenic animals was consistently observed when the *odr-1p::sax-7shortcDNA* was injected at 300 ng/ μ l, which likely gave rise to very high expression of short form SAX-7 protein in both AWB and AWC neurons. This forced attachment was variably detected between different transgenic strains, when the *odr-1p::sax-7shortcDNA* was injected at 5 or 50 ng/ μ l (data not shown). These results suggest that the almost artificially high level of SAX-7 expression may be required for induction of homophilic adhesion *in vivo*. We thus cannot rule out the possibility that the forced attachment is due to overexpression of the injected transgene and does not reflect normal homophilic adhesive activity *in vivo*. Nonetheless, these results certainly implicate potential homophilic adhesion ability of SAX-7 *in vivo*.

Discussion

In this study, we report that SAX-7, a *C. elegans* homologue of neural cell adhesion molecule L1, is required for maintenance of neuronal positions and the organization of ganglia. The short form of SAX-7 showed stronger rescuing ability for neuronal positioning than the long form, which is consistent with much stronger homophilic adhesive activity of short form than the long form *in vitro*. Expression of SAX-7 in a single neuron of *sax-7* mutants cell-autonomously restored neuronal positions, implicating the existence of SAX-7-mediated heterophilic adhesion system. Expression of the short form SAX-7 in two separated head neurons in *sax-7* null mutants induced unusual cell-cell attachment *in vivo*. These results suggest that both homophilic and heterophilic interaction of SAX-7 *in vivo* may play roles in the maintenance of neuronal positions and organization of ganglia (Figure 8A). We also showed that abnormal AFD positions of *sax-7(nj48)* mutants were restored in *unc-54(e190)* mutant background where the body movement is severely defective (Table I). Thus, the suppression of abnormal neuronal positions by body paralysis suggests that the maintenance of neural positions by SAX-7 is required for antagonizing mechanical force accompanied with the movement. Overall, we

Figure 7 SAX-7 exhibits both heterophilic and homophilic adhesion *in vivo*. (A–E) Heterophilic adhesion *in vivo*. AWB and AWC were labeled with GFP, and AFD was labeled with dsRed2 (pseudocolored to magenta). (A) In wild type, AWB, AWC, and AFD were all localized posterior to the nerve ring. (B) In *sax-7(nj48)* mutants, AWB, AWC, and AFD were all mislocalized anterior to the nerve ring. (C) *sax-7shortcDNA* was expressed only in AFD in *sax-7(nj48)* mutant background. The position of AFD was restored to be posterior to the nerve ring, but AWB and AWC were still mislocalized anterior to the nerve ring. White, yellow, and magenta arrows indicate the cell body of AWB, AWC, and AFD, respectively. An arrowhead indicates the nerve ring. Scale bar is 20 μm. (D) Quantitative analysis of cell body positions in *sax-7(nj48);Ex[gcy-8p::sax-7shortcDNA, odr-1p::GFP, gcy-8p::dsRed2]* injected at 300 ng/μl. For each cell type, > 300 neurons were counted. (E) Dose dependency of the restoration of AFD positions. (F–I) Homophilic adhesion *in vivo*. (F) In wild type, AWB and AWC are spatially separated. (G) In *sax-7(nj48)* mutants, although AWB and AWC were mislocalized anteriorly, they were still spatially separated. (H) Expression of *sax-7shortcDNA* both in AWB and AWC in *sax-7(nj48)* mutant background induced cell–cell attachment. Photos are the tight attachment of AWB and AWC observed in two independent animals. A white arrow indicates the cell body of AWB and a yellow arrow indicates the cell body of AWC. Scale bar is 20 μm. (I) Quantification of two cell attachment of AWB and AWC.

propose that SAX-7-mediated adhesion is a fundamental system to insure the maintenance of neuronal order.

Maintenance of neuronal positions by SAX-7/L1 cell adhesion system

Although how neuronal positions are maintained after the generation of nervous system is an important question, little

is known about molecular basis for maintenance of neuronal positions. Our genetic approach, using *C. elegans*, revealed that L1 molecule is important for the maintenance of neuronal positions. Given that the functions of Ig molecules, for example UNC-40/DCC and SAX-7/Robo, are highly conserved in the nervous system from worm to vertebrate (Chan *et al*, 1996; Zallen *et al*, 1998; Rougon and Hobert, 2003), it is

Figure 8 Proposed model for SAX-7-mediated neuronal adhesion *in vivo*. (A) Heterophilic adhesion to other tissues or cells may play an important role in maintenance of neuronal positions, and homophilic adhesion of SAX-7 between neighboring neurons may contribute to the organization of ganglia. (B) Model for different adhesive activities of short and long SAX-7. Short form may correspond to the opened state and show strong adhesion. Alternatively, long form could undertake reversible states, the opened and closed (horseshoe) states, and the closed state could produce inhibitory effect for adhesion.

highly likely that L1 molecules have conserved maintenance role in vertebrate nervous system. The recent study of CHL (Close Homologue of L1) showed that layer-specific neuronal positioning in the cerebral cortex is abnormal in CHL knockout mouse (Demyanenko *et al*, 2004). The close examination of impaired neurons in CHL1, L1CAM, or NrCAM knockout mice at the later stage could possibly reveal abnormalities in maintenance of neuronal positions.

Homophilic and heterophilic adhesion of SAX-7 in organization of ganglia and maintenance of neuronal positions

Neuronal cell bodies in the ganglia around the nerve ring are localized in the space between the pharynx and the hypodermis. The spatial relationship between neurons and the pharynx is rigorously maintained when body size becomes larger as animals grow. The homophilic interactions of SAX-7 could possibly explain the organization of ganglia, but cannot fully explain the maintenance of neuronal positions during the worm growth. Unless the organized ganglia are attached, they would float and wander when the animals move. Heterophilic adhesion of SAX-7 with other molecules could

ensure the maintenance of neuronal positions during the movement. Consistent with this assumption, the cell-autonomous rescue of neuronal positioning by expressing SAX-7 in a single neuron implies the existence of a heterophilic partner of SAX-7 (Figures 7A–E and 8A). It is possible that neurons at medial positions attach to the basement membrane of the pharynx, neurons at lateral positions attach to the hypodermis, and neurons at intermediate positions attach with each other or extracellular matrix between the pharynx and the hypodermis. Cell adhesion molecules of L1 family were reported to interact with proteins such as integrins, laminins, and proteoglycans *in vitro* (Hortsch, 1996, 2000; Grumet, 1997; Brummendorf *et al*, 1998; Brummendorf and Lemmon, 2001). Likewise, SAX-7 could interact with these molecules.

The different adhesion activities between SAX-7 long form and short form could be explained by the differences in three-dimensional structure

The SAX-7 short form had stronger rescuing activity for neuronal positioning *in vivo* and stronger homophilic adhesion activity *in vitro* than the long form. Recent studies on three-dimensional structures of L1 and L1-related proteins predicted that Ig-like domains of these proteins take horseshoe structures, namely the initial two Ig-like domains fold back along the third and fourth Ig-like domains (Su *et al*, 1998; Freigang *et al*, 2000; Schurmann *et al*, 2001). Accordingly, a molecular model was proposed in which the L1 protein changes its conformation between an open state with high adhesion activity and a closed (horseshoe) state with low activity *in vivo* (Su *et al*, 1998). Based on this model, the SAX-7 long form with six Ig-like domains could form a horseshoe and inhibited structure, whereas the short form with four Ig-like domains could only form an open and strong adhesive structure (Figure 8B). It is plausible to predict that the difference of adhesive activity of both forms is due to the difference of structural states.

Two L1 homologues in *C. elegans* genome

The cell biological studies of L1 molecules and genetic and anatomical analysis of knockout mouse or human patients demonstrated the importance of L1 molecules in axonal growth and guidance (Jouet *et al*, 1994; Hortsch, 1996, 2000; Van Camp *et al*, 1996; Burden-Gulley *et al*, 1997; Grumet, 1997; Brummendorf *et al*, 1998; Kamiguchi *et al*, 1998; Kenwrick and Doherty, 1998; Brummendorf and Lemmon, 2001). The recent study showed that crosstalk with L1 and Neuropilin is important for axonal pathfinding (Castellani *et al*, 2000, 2002; Rougon and Hobert, 2003). The drastic abnormalities of axons were not observed in *sax-7* mutants (Zallen *et al*, 1999). Besides SAX-7, there is another homologue of L1 in *C. elegans* genome, Y54G2A.25 (Aurelio *et al*, 2002; Vogel *et al*, 2003) (Figure 2D). Thus, the other L1-like molecule might play an important role in axonal pathfinding in *C. elegans*. Alternatively, it is possible that SAX-7 and the other L1-like molecule redundantly function for axonal development. Our sequence analysis indicates that SAX-7 is more similar to mammalian NrCAM rather than L1CAM. In addition, the sequence at the C-terminus of SAX-7 is PDZ binding motif (T-F-V) that is conserved in NrCAM and not in L1CAM (Grumet *et al*, 1991; Kayyem *et al*, 1992). The high level of expression of NrCAM in mature brain (Davis *et al*, 1996) is consistent with the possibility that NrCAM has

an important role in maintenance of the nervous system. Further studies on the SAX-7-mediated adhesion system, such as the identification of heterophilic partners and downstream molecules, may shed light on the molecular mechanism of L1-mediated neuronal adhesion.

Materials and methods

C. elegans molecular genetics

C. elegans strains were maintained using standard methods (Brenner, 1974). We introduced AFD- and AIY-specific GFP markers into the mutator strain *mut-7(pk204)* for visual mutant screening (Hobert *et al*, 1997; Ketting *et al*, 1999). *sax-7(nj13)* and *sax-7(nj52)* were isolated as exhibiting abnormal positioning of AFD and AIY neurons. *sax-7(ky146)* was kindly provided by C Bargmann and J Zallen (Zallen *et al*, 1999). We cloned the *sax-7* gene by the transposon display technique (Korswagen *et al*, 1996; Wicks *et al*, 2000). Transformation with cosmid clone C18F3 that covered the predicted *sax-7* gene rescued the abnormal neuronal positioning of *nj13* mutants. Deletion alleles *nj47*, *nj48*, and *nj53* were isolated in PCR screens of a deletion mutant library (~24 000 000 genomes) generated by TMP/UV method (Yandell *et al*, 1994; Gengyo-Ando and Mitani, 2000). All *sax-7* mutants were outcrossed more than six times with wild type. *nj47* mutants showed larval arrest and uncoordinated phenotype. Since these abnormalities were not rescued by the cosmid clone C18F3 that covers the whole *sax-7* locus and *nj53* mutants carrying the similar deletion did not show any morphological defects, morphological defects of *nj47* mutants are likely to be caused by defect in the closely positioned gene(s).

Isolation of cDNAs and construction of full-length cDNAs

The 3'-partial *sax-7* cDNA was generously provided by L Chen (Chen *et al*, 2001). 5'-cDNAs were isolated by screening a cDNA library and by use of RT-PCR. Two forms of 5'-cDNA, long and short forms, were isolated. The full-length cDNAs of both long and short forms were constructed by ligation with the 3'-cDNA.

Construction of *sax-7cDNA::GFP*

The *gfp* gene and the 3'-UTR of *sax-7* were inserted into the carboxyl terminus of a full-length *sax-7* short or long cDNA (Shen and Bargmann, 2003). *sax-7* cDNAs were ligated to the *osm-6* or *gcy-8* promoters.

Western blotting

C. elegans whole lysates were prepared and were diluted into SDS-PAGE sample buffer with 2% β -mercaptoethanol. Equal amounts of total protein from each lysate were used for Western blotting. In addition to 160 and 140 kDa bands, 45 kDa band was detected in the wild-type sample (Chen *et al*, 2001), which was undetected in *sax-7(nj48)*, *sax-7(nj13)*, *sax-7(ky146)*, and *sax-7(nj52)* mutants. This 45 kDa band is not the product of another splicing variant, but the product of protein cleavage of long and short SAX-7 protein, because we detected the 45 kDa band in *sax-7(nj48)* animals carrying cosmid clone C18F3, *unc-14p::longcDNA*, or *unc-14p::shortcDNA*.

Transgenic experiments

osm-6p::GFP, *glr-1p::GFP*, *unc-14p::GFP*, *H13p::GFP*, *ttx-3p::GFP*, *odr-1p::GFP*, or *glr-3p::GFP* was used (Hart *et al*, 1995; Maricq *et al*, 1995; Hobert *et al*, 1997; Ogura *et al*, 1997; Yu *et al*, 1997; Collet *et al*, 1998; L'Etoile and Bargmann, 2000; Brockie *et al*, 2001). These GFP markers were injected at 10–50 ng/ μ l (Mello *et al*, 1991). To generate *sax-7* expression constructs, *gcy-8*, *odr-1*, or *unc-14* promoters were fused to *sax-7* full-length cDNAs. For rescue

experiments, the *unc-14p::sax-7* short form cDNA was injected at 5 ng/ μ l and the *unc-14p::sax-7* long form cDNA was injected at 0.5, 5, or 50 ng/ μ l into *sax-7(nj48)* mutants.

Cell-autonomous rescue experiments

gcy-8p::sax-7 short form cDNA was injected at 5, 50, 150, 300, and 500 ng/ μ l with *gcy-8p::dsRed* and *odr-1p::GFP* or *gcy-8p::GFP* and *odr-1p::RFP* into *sax-7(nj48)* mutants. At least three independent lines were scored for cell body positions of AFD, AWB, and AWC neurons on the same lateral side.

In vivo cell-cell attachment experiments

To address whether SAX-7 could homophilically interact *in vivo*, *odr-1p::sax-7* short form cDNA was injected at 5, 50, and 300 ng/ μ l with *odr-1p::GFP*. The attachment of AWB and AWC cell bodies on the left or right lateral side was scored in more than four independent lines using an Olympus Fluoview confocal microscope.

Generation of SAX-7-expressing cells by silkworm baculovirus expression

Subcloned long or short form *sax-7* cDNA was cleaved with *NotI* and *SalI*. cDNA fragments were inserted into pYNG-sig, which was kindly provided by T Suzuki. The constructed plasmid was mixed with purified viral DNA (Maeda *et al*, 1985). The mixture was cotransfected in BmN4 cells of the silkworm *B. mori* larvae using the Lipofectin reagent (Invitrogen). Recombinant virus was screened by the end-point dilution methods in 96-well plates, and a polyhedrin negative clone was obtained. The recombinant virus was propagated and concentrated on BmN4 cells. BmN4 cells were cultured for 4 days after infected with these recombinant viruses, checked for SAX-7 protein expression, and used for further analysis in aggregation assays.

Measurement of cell aggregation

A total of 1×10^6 cells suspended in 3 ml of HCMF buffer were put into each well in 24-well plates, which had been coated with BSA to prevent attachment of cells to the dishes. Cells were incubated at 27°C on a gyratory shaker at 60 r.p.m. To measure cell aggregation, the total particle number in cell suspension was counted. The extent of aggregation was represented by the ratio of the total particle number at time *t* (60 min) of incubation (N_t) to the initial particle number (N_0), the latter being identical to the total number of cells added to the medium (Takeichi, 1977).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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