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·Original Article·

HLB-1 functions as a new regulator for the organization and function of neuromuscular junctions in nematode *Caenorhabditis elegans*

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Abstract: Objective To study the role of HLB-1 in regulating the organization and function of neuromuscular junctions in nematode *Caenorhabditis elegans*. **Methods** To evaluate the functions of HLB-1 in regulating the organization and function of neuromuscular junctions, effects of *hlb-1* mutation on the synaptic structures were revealed by uncovering the expression patterns of SNB-1::GFP and UNC-49::GFP, and pharmacologic assays with aldicarb and levamisole were also used to test the synaptic functions. Further rescue and mosaic analysis confirmed HLB-1's role in regulating the organization and function of neuromuscular junctions. **Results** Loss of HLB-1 function did not result in defects in neuronal outgrowth or neuronal loss, but caused obvious defects of SNB-1::GFP and UNC-49::GFP puncta localization, suggesting the altered presynaptic and postsynaptic structures. The mutant animals exhibited severe defects in locomotion behaviors and altered responses to an inhibitor of acetylcholinesterase and a cholinergic agonist, indicating the altered presynaptic and postsynaptic functions. Rescue and mosaic analysis experiments suggested that HLB-1 regulated synaptic functions in a cell nonautonomously way. Moreover, HLB-1 expression was not required for the presynaptic active zone morphology. Genetic evidence further demonstrated that *hlb-1* acted in a parallel pathway with *syd-2* to regulate the synaptic functions. **Conclusion** HLB-1 appeared as a new regulator for the organization and function of neuromuscular junctions in *C. elegans*.

Keywords: HLB-1; synaptic function; neuromuscular junction; SYD-2; *Caenorhabditis elegans*

1 Introduction

Chemical synapses provide information flow between presynaptic and postsynaptic, and transmitter release is supported by specialized subcellular structures developing at synaptic junctions[1,2]. Active zones at the presynaptic termini provide the structural basis of the exocytosis and endocytosis with electron-dense structures[3,4], and mediate Ca^{2+}-triggered neurotransmitter release from synaptic vesicle fusion[5]. Morphological elucidation of the active zone suggests that a protein scaffold organizes the active zones and links it physically to presynaptic vesicles[6]. To date, mainly three active zone proteins have been identified in *Caenorhabditis elegans* as the synaptic scaffolds: UNC-10/RIMs are putative Rab3A-effectors in regulating synaptic vesicle exocytosis and neurotransmitter release[7], Unc-13/Munc-13s are required for priming synaptic vesicles for exocytosis and synaptic transmission[8], and α-liprins/SYD-2 bind to and recruit tyrosine phosphatases (receptor type) to defined areas for active zones development and normal active zone function[9-11].

Being a family of LAR transmembrane protein-tyrosine phosphatase interacting proteins, liprins are subdivided into

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two types: α-type and β-type[12]. As a homologue of α-liprin, SYD-2 regulates the presynaptic differentiation in *C. elegans*, especially the active zone formation[9]. However, aldicarb pharmacologic assay revealed that unlike other active zone proteins, such as UNC-10 or UNC-13, SYD-2 null only induced mild locomotion behavior defect and moderate impairment of neurotransmission, suggesting that there might be another liprin homologue that played redundant roles at presynaptic. *hlb-1* (homologue of β-liprin) encodes the only liprin protein in *C. elegans*. In human cells, the C-terminal non-coiled-coil region of liprin α binds to the C-terminal non-coiled-coil region of liprin β[9], suggesting that SYD-2 and HLB-1 may bind together biochemically and act as a complex in controlling the presynaptic differentiation. In this study, our results suggest that HLB-1 may play an essential role in regulating the organization of neuromuscular junctions in *C. elegans*.

2 Materials and Methods

2.1 Strains and culture conditions *C. elegans* culture and manipulation were performed using standard methods[13]. All strains were cultured at 20°C on NGM (nematode growth media) plates with *Escherichia coli* OP50. Strains used in this work were obtained from the Caenorhabdits Genetics Center (funded by the NIH National Center for Research Resource). The deletion allele *ok725* for *hlb-1* was identified by nest-PCR amplification with primers of RB1 (5'-CCATTCGTATGGTGTGCAAG-3'), RB2 (5'-ACGCATTATTCGGATTCTGG-3'), RB3 (5'-CATGGTCCATTTCGTTCTGA-3'), and RB 4 (5'-AACAGGAGTGCCCACGTTAC-3'), which span the deleted region. The deletion fragment was further purified and sequenced, and the homozygous deletion strain RB878, was backcrossed with N2 wild-type animals three times.

2.2 DNA construct The right end (the ApaI end of *hlb-1*) of cosmid T21H8 (obtained from the Sanger Institute, Cambridge, UK) containing *T21H8.1 (hlb-1)*, is close to the SacI site of Lorist2 vector. A 10.0-kilobase (kb) BamHI/ApaI fragment from T21H8 was cloned into pSL1180 vector (Promega) to generate the clone pDY02. A 3.8 kb BamHI/StuI fragment from T21H8 was also cloned into the pSL1180 vector to generate clone pDY03. The 3.8 kb BamHI/StuI fragment from cosmid T21H8 was further inserted into the BamHI/StuI sites of plasmid pDY02 to generate clone pDY05. The DNA constructs were summarized in Fig. 5. Transgenic worms were generated as previously described[14]. The marker *rol-6* (plasmid pRF4) and constructs were coinjected into gonads of hermaphrodite animals.

2.3 Behavior assay Locomotion behavior assay was performed as previously described[15]. A thrash was defined as a change in the direction of bending at the mid body. A body bend was counted as a change in the direction of the part of the nematodes corresponding to the posterior bulb of the pharynx along the *y* axis, assuming that the nematode was traveling along the *x* axis. Pharynx pumping rates were scored on adults at room temperature as described[16]. As previously reported[17], brood size was assessed by the presence of larvae after placing single tested animal onto individual well of tissue culture plates. Thirty nematodes were examined per treatment. The simple chemotaxis to NaCl (100 mmol/L) was deteced as described by Bargmann and Horvitz (1991)[18]. As a control, sodium azide was also spotted approximately 4 cm away from the center of the NaCl gradient. Approximately 100 animals were included in each treatment. The percentage of animals performing IT (Isothermal tracking) was determined by the tracks left on the agar surface after removal of the animals from the plate as described[19]. All assays were more than triplicated.

2.4 Microscopy and quantification Live epifluorescence microscopy was operated as described[9]. Fluorescent and Nomarski images were obtained using a Zeiss Axiophot microscope equipped with a Photometrics Sensys CCD camera. Quantification of SNB-1::GFP, UNC-49::GFP and UNC-10 expressions was performed as described[20-22].

2.5 RNA blotting Total RNA was isolated using guanidinium thiocyanate/phenol method as described[23]. The frozen worm (strains of N2 and *hlb-1(ok725)*) pellets were harvested from 1 liter liquid cultures at mixed stages. Poly $(A)^+$ RNA purified after two rounds of selection on oligo(dT)-cellulose (Collaborative Biomedical Products type 3 or type 2) was used for the RNA blotting. Primers were as follows: hlb-1a 5'-CTTTTCGTTGATCCATCGGT-3', h1b-1b 5'-

CCAACAACAACTTGCACACC-3'; syd-2a 5'-ATGAGCTACAGCAATGGAAACATA-3', syd-2b 5'-ACATTCCGTTTACATTGTTTTCTA-3'; unc-10a 5'-TCCCATTTATCTGCAGAAGAACGT-3', unc-10b 5'-TTGTTGCCAATGCTCAAGAAAGAA-3'. PCR was performed at 94 ºC for 45 s, 60 ºC for 45 s, 72 ºC for 90 s, then repeated 30 cycles. rRNA was used to determine the loading amount for each sample.

2.6 Immunohistochemistry Immunohistochemistry was performed as described[7]. The antibody in this report was anti-UNC-10 (1:1000)[7,22].

2.7 Pharmacologic assay Levamisole-induced egg-laying were performed as described[24]. Aldicarb resistance assays were done as described[25]. Briefly, adult animals were examined for drug sensitivity through body movement or pharyngeal pumping after being exposed to the indicated concentrations of aldicarb for 8 h. Animals were classified as positive in pharyngeal pumping if they demonstrated continuously, and positive in movement if any of their body wall muscle activities could be observed. Twenty to thirty worms per plate were monitored. Graphs represented at least ten trials.

2.8 Mosaic analysis Mosaic analysis was performed as previously reported[9] using nuclear SUR-5::GFP as a marker[26]. Three animals that had lost the array from the muscle lineage and four animals that had lost the array from the neural lineage were examined for *juIs1* expression pattern.

2.9 Genetics Double mutant constructs of *hlb-1(ok725) syd-2(ju37)* and *hlb-1(ok725)syd-2(ju37);juIs1* were carried out using standard genetic protocols. The homozygous double mutant worms were identified from the *syd-2*(*ju37*) behavior phenotype of slightly backing up, the *syd-2*(*ju37*); *juIs1* marker, and the PCR screen for F3 progeny of the *hlb-1* deleting worms.

2.10 Statistical analysis Data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by a Dunnett's *t*-test was used to determine the significant difference between the groups. $P<0.05$ was considered as statistically significant.

3 Results

3.1 Identification of a *hlb-1* deletion HLB-1 has a coiled-coil domain at amino acids 278-326 and a SAM domain (Sterile alpha motif) at amino acids 728-792 (Fig. 1A), indicating that *hlb-1* encodes a SAM domain-containing liprin protein in *C. elegans*. The SAM domain could form extended polymeric structures based on the presence of two distinct intermononers binding surfaces, suggesting that HLB-1 may function as another scaffold component during synaptogenesis. Furthermore, HLB-1 shows highly homologue in coiled-coil domain (31% identity) and SAM domain (39% identity) with liprin β1 (Fig. 1B), suggesting the pos-

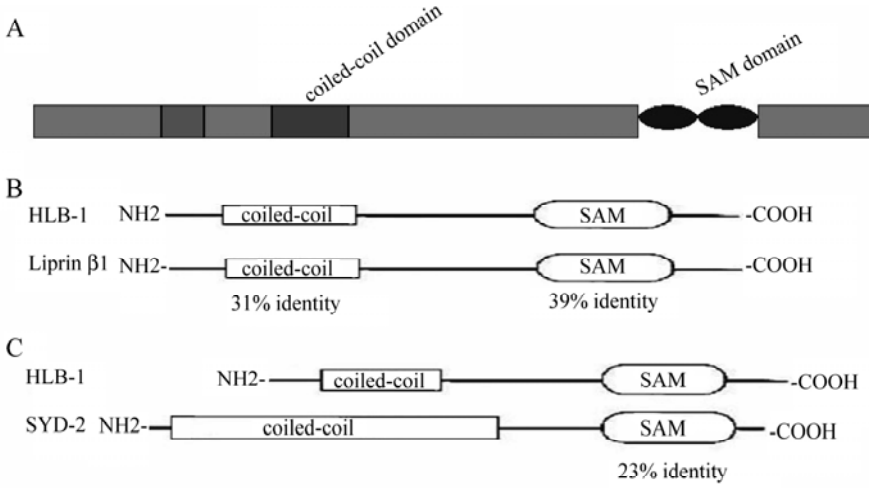

Fig. 1 ***hlb-1*** **gene structure. A: Motif analysis of HLB-1 protein. Domains of ncoils and SAM are indicated in the schematics. B: Protein comparison of HLB-1 and mammalian liprin β1. ClustaIX was used as the alignment tool. C: Protein comparison of HLB-1 and SYD-2. ClustaIX was used as the alignment tool.**

sible similar biological function of HLB-1 with liprin β1. However, there shows no significant homologue between HLB-1 and SYD-2 at their amino terminals, especially at the coiled-coil domain, although high homologue exists at their SAM domains (23% identity, 42% similarity) (Fig. 1C). Therefore, HLB-1 may have new and different functions from SYD-2.

To investigate the possible functions of HLB-1 in synaptogenesis, especially in addressing the SYD-2 function at presynaptic, we first confirmed a deletion strain of *hlb-1 (ok725)* from Caenorhabdits Genetics Center (funded by the NIH National Center for Research Resource). Results showed that *hlb-1 (ok725)* caused an approximately 800-900 bp fragment deletion (Fig. 2A). The *hlb-1* fragment amplified from the *hlb-1 (ok725)* DNA was sequenced with RB3 (inner left primer) and RB4 (inner right primer) as sequencing primers, and *hlb-1 (ok725)*, with a deletion of 836 bp fragment, resulted in the loss of most of the coiled-coil domain (Fig. 3A). In addition, RNA blotting further confirmed the loss of *hlb-1* function in *hlb-1 (ok725)* deletion mutant (Fig. 2B).

3.2 Loss of *hlb-1* function affects nematode behaviors Using this deletion strain, the effects of *hlb-1* on behavior in *C .elegans* were next examined. The behavior of *hlb-1 (ok725)* animals was similar with wild type, but different from *syd-2 (ju37)* animals, that exhibited slightly backing up phenotype (Fig. 3B). In addition, mutation of *hlb-1* did not result in obvious chemotaxis defects to NaCl (Fig. 3C), lysine or biotin (data not shown), nor in the thermotaxis defects (Fig. 3D). However, the deletion of *hlb-1 (ok725)* caused significant *(P* < 0.01) impairment in the speed of straight stinusoidal locomotion as quantified by measuring the body bends per time interval (Fig. 3E) and a noticeable (*P* < 0.01) decrease in frequency of thrashing in liquid solution (Fig. 3F) for both the L1 larvae and adult nematodes compared to wild-type. Since neurons and muscle cells need to form correct cellular connections and assemble a specific repertoire of signaling proteins into synaptic structures, disruptions in any of these steps would induce defects of locomotion behavior[13]. The effects of *hlb-1* deletion on locomotion behaviors suggest that *hlb-1* may be involved in the regulation of synaptic function and ensure appropriate contacts between neurons and muscle cells. Moreover, mutation of *hlb-1* significantly (*P* < 0.01) reduced the pumping rate at day 8 and 10 (Fig. 3G) and brood size (Fig. 3H), suggesting that HLB-1 may also be involved in the control of aging and reproduction in *C. elegans*.

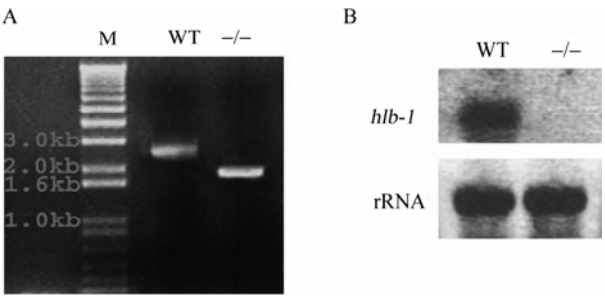

Fig. 2 Identification of a deletion of *hlb-1*. A: PCR analysis of the size of deletion fragment. WT, N2 wild-type; -/-, *hlb-1 (ok725)*. M, 1 kb DNA ladder. B: Effects of deletion on the *hlb-1* transcription. Total RNAs from adult worms under normal conditions were used for RNA analysis. rRNA was used as an equal loading control.

3.3 Loss of *hlb-1* function will not result in defective neuronal outgrowth and neuronal loss Previous studies indicated that SYD-2 and HLB-1 expressions appeared predominantly in neurons and muscle cells, with co-expression in pharyngeal and body wall muscle[9-12]. To test whether the observed abnormal behavioral phenotypes in *hlb-1* mutant was caused by defects in the development of nervous system, we further characterized the architecture of the motor neurons in *hlb-1* null animals. The GABA nervous system in *hlb-1(ok725)* adult animals was indistinguishable from that in the wild-type as revealed by a green fluorescence protein (GFP) tag (Fig. 4A). Similarly, no obvious changes of pan-neural vesicle marker synaptogyrin-GFP (*jsIs219*) were observed in *hlb-1(ok725)* mutant compared to wild-type (data not shown). Moreover, in *hlb-1(ok725)* mutant animals, the axon of AFD sensory neuron terminated at the proper position as observed in wild-type animals (Fig. 4B). The sensory ending of AFD neuron in *hlb-1(ok725)* mutant animals was also normal (Fig. 4B). In addition, the axons could extend along both the ventral and dorsal nerve cords of GABA nervous system and were stably maintained in *hlb-1(ok725)* adults animals (data not shown). Furthermore, no noticeable absence of GABAergic inhibitory motor neurons ($VD_{1\text{-}13}$ and

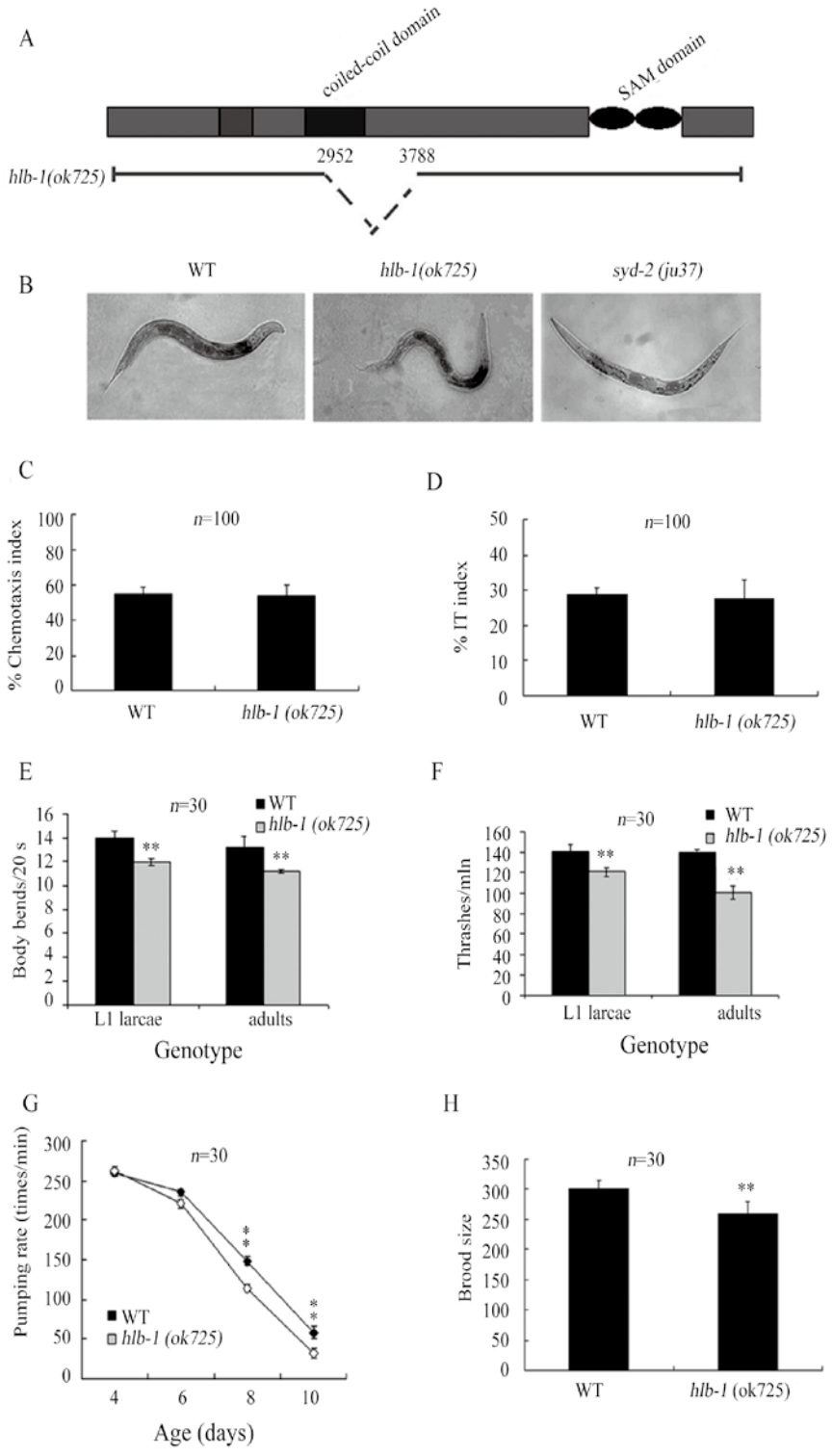

Fig. 3 Effects of the deletion of *hlb-1* on nematode behaviors. A: *hlb-1 (ok725)* caused the deletion of most of the coiled-coil domain. 2592 and 3788 represent nucleotides number. B: Comparison of the behavior phenotype. C: Comparison of chemotaxis to NaCl. D: Comparison of isothermal tracking (IT). E: Comparison of body bends. F: Comparison of head thrashes. G: Comparison of pumping rate during development. H: Comparison of brood size. WT, wild-type N2. Error bars indicate SEM. **$P < 0.01$.

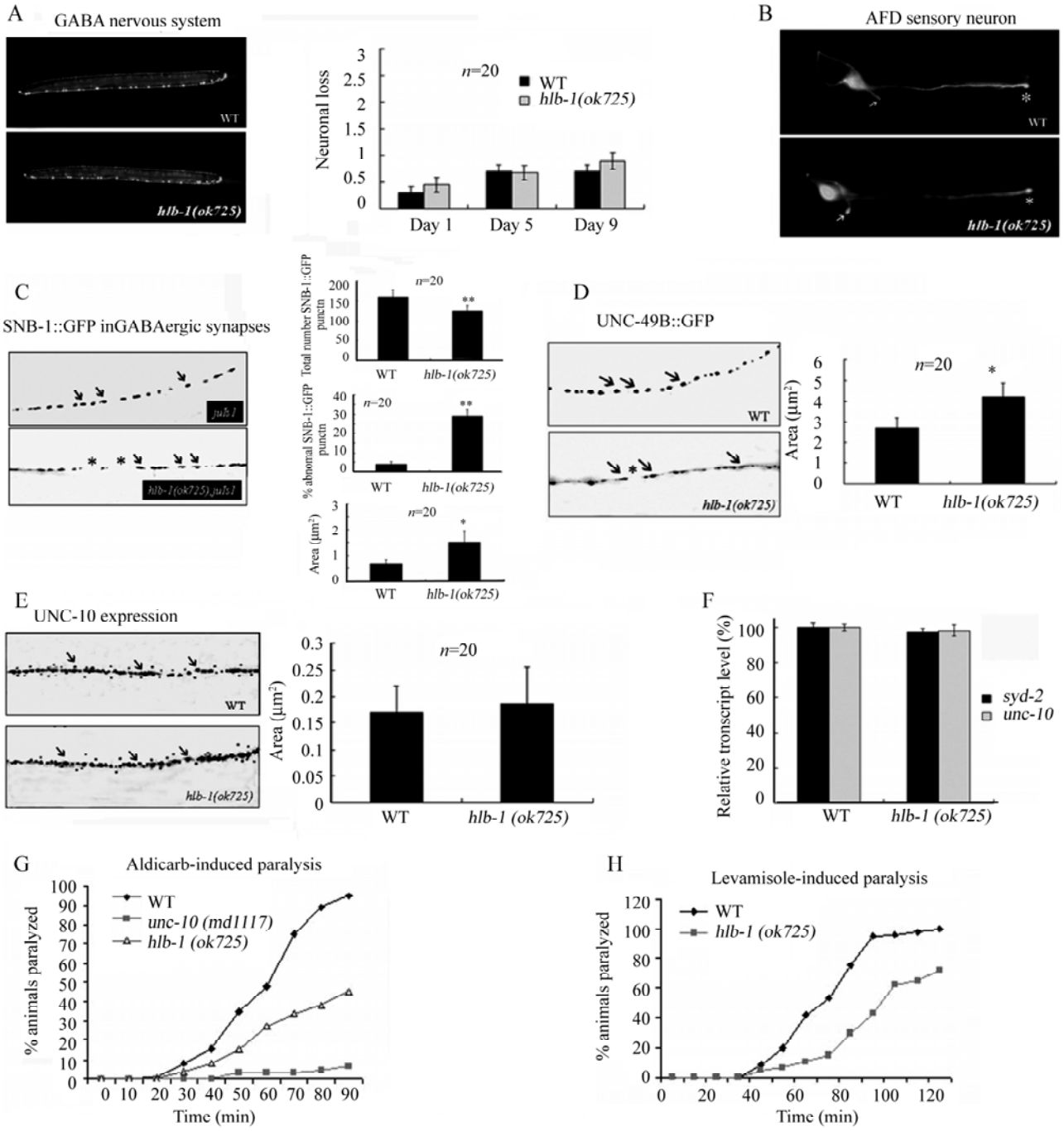

Fig. 4 Deletion of *hlb-1* regulated organization of neuromuscular junctions. A: GABA motor neuron anatomy visualized using *oxIs12* was normal and no neuronal loss was observed in *hlb-1* null mutant. Dorsal is up, anterior is right. B: Reciprocal axon phenotypes. Anterior is right. AFD neurons were visualized by *lin-15(n765); dEx1267*. Arrows indicate axons, and asterisks indicate sensory endings. C: The dorsal cords of GABAergic presynaptic terminals visualized by *juIs1*. To quantify SNB-1::GFP puncta morphology, abnormality was scored as percentage of irregularly shaped puncta. Arrows indicated fluorescent puncta, and asterisks indicated fluorescent gap. D: Distributions of postsynaptic GABA receptor clusters in the muscles visualized by *oxIs22*. Arrows indicated fluorescent puncta, and asterisks indicated fluorescent gap. E: Expression of UNC-10. Arrows indicated the UNC-10 fluorescent puncta. F: Comparison of transcript levels of *syd-2* and *unc10*. G: Aldicarb-induced paralysis. *unc-10 (md1117)* was used as a control having high resistance to aldicarb. H: Levamisole-induced paralysis. WT, wild-type N2. Error bars indicate SEM. *$P < 0.05$, **$P < 0.01$.

$DD_{1\text{-}6}$) during development was detected in *hlb-1(ok725)* mutant animals (Fig. 4A). These data suggest that the primary defect associated with the loss of HLB-1 function is not due to a defect in neuronal outgrowth or neuronal loss.

3.4 Loss of *hlb-1* function causes the neuromuscular junction (NMJ) defects In wild-type animals, the *unc-25* promoter drives the uniformly shaped fluorescent SNB-1::GFP puncta to be evenly distributed along the ventral and dorsal cords, which corresponds to the presynaptic termini of 13 VD and 6 DD neurons, respectively. However, further examination of individual presynaptic zone of GABAergic motor neuron synapses using the P*unc-25*-SNB-1::GFP (*juIs1*) revealed an abnormal SNB-1::GFP puncta in the *hlb-1(ok725)* mutants, which appeared slightly diffused and were usually more widely spaced than normal, together with a decrease in the number of puncta in both ventral and dorsal cords (Fig. 4C). Unlike the *syd-2(ju37)* animals that showed the same total number of SNB-1::GFP as wild-type[9], *hlb-1(ok725)* animals displayed an average of only 125 ventral and 121 dorsal puncta, as compared with the ~154 SNB-1::GFP puncta in both the ventral and dorsal cords in wild type animals. Moreover, percentage of abnormal SNB-1::GFP puncta in *hlb-1(ok725)* (29 ± 3.5) was about 7 folds of that in wild-type animals (4 ± 1.2) (Fig. 4C). We further calculated the area of the presynaptic zones in wild-type and *hlb-1(ok725)* mutant animals, and observed that the area of the presynaptic zone in *hlb-1(ok725)* (1.5 ± 0.45) was significantly (P <0.05) enlarged compared to that in wild-type animals (0.8 ± 0.14) (Fig. 4C). These findings indicated that HLB-1 was involved in the regulation of presynaptic function, and that loss of *hlb-1* function could cause different synaptic defects from *syd-2* null mutation at presynaptic.

Since one essential property of synapses is to precisely register the presynaptic and postsynaptic structures[21], we further asked whether the presynaptic defects in *hlb-1 (ok725)* mutant were correlated with defects in the postsynaptic structures in muscle cells. As shown in Fig. 4D, the distribution of GFP-tagged GABA receptor in *hlb-1(ok725)* was distinguishable from that in wild-type. In *hlb-1(ok725)* mutant the puncta appeared enlarged, diffused, and spaced farther apart than normal, and the area of the postsynaptic zone in *hlb-1(ok725)* (4.2 ± 0.7) was also significantly (P < 0.05) enlarged compared to that in wild-type animals (2.7 ± 0.5) (Fig. 4D). Therefore, both presynaptic and postsynaptic structures were affected in *hlb-1(ok725)* mutant animals, and the defects in the pattern of the UNC-49::GFP marker was correlated with those seen with the SNB-1::GFP marker in *hlb-1(ok725)* mutant animals.

3.5 Loss of *hlb-1* function does not affect the active zone formation Previous ultrastructural analysis revealed that the active zones of *syd-2(ju37)* mutants could be significantly lengthened, which suggested that SYD-2 might play a pivotal role in controlling active zone formation in *C. elegans*[9]. We next asked whether mutations in *hlb-1(ok725)* animals would also result in defects of the formation of presynaptic active zone. In both wild-type and *hlb-1(ok725)* animals, expression patterns of UNC-10 were examined by immunohistochemistry assay, since UNC-10 was known as an active zone protein and its expression patterns reflected the change of presynaptic active zone morphology[7]. However, no differences for the UNC-10 localization at active zones could be found, since the UNC-10 puncta were evenly sized and spaced along the nerve cords in *hlb-1(ok725)* mutant as observed in wild-type (Fig. 4E). In addition, the area of UNC-10 puncta in *hlb-1(ok725)* (0.19 ± 0.07) was also similar with that in wild-type (0.17 ± 0.05) (Fig. 4E). Thus, loss of *hlb-1* function will not affect the active zone morphology, but may influence synaptic function at presynaptic, which means that the function of HLB-1 is relatively specific and different from that of SYD-2 during synaptogenesis. Moreover, RNA blot results indicated no difference of *unc-10* or *syd-2* transcriptional expression level between wild-type and *hlb-1(ok725)* animals (Fig. 4F).

3.6 *hlb-1 (ok725)* display a decreased sensitivity to cholinergic agonist and antagonists Because the synaptic transmission was only partially impaired in *syd-2* mutants[9], we assumed that the mutation of *hlb-1* might result in severe defects of synaptic transmission if HLB-1 could address the SYD-2 function at presynaptic. To assess this synaptic functional defects, two neuroactive drugs, aldicarb and levamisole, were used to dissect the neuromuscular signaling in *C. elegans*[27,28], and their response to the chlolinesterase in-

hibitor aldicarb was first analyzed. As previous studies indicated[27], mutations in neurotransmission at the presynaptic site that caused a less pronounced accumulation of acetylcholine, induced resistance to aldicarb to different degrees, since aldicarb inhibited acetyl cholinesterase (AChE), resulting in an accumulation of acetylcholine, overstimulation of acetylcholine receptors and paralysis of body wall muscles in animals. Compared to the wild-type, *hlb-1 (ok725*) animals were highly resistant to aldicarb (Fig. 4G), suggesting that mutation of *hlb-1* resulted in the neurotransmission defects at presynaptic. To further assess whether the synaptic transmission was altered in *hlb-1 (ok725)* mutants, the response to the acetylcholine agonist levamisole was also measured. Sensitivity to the acetylcholine receptor agonist levamisole will persist if the mutation is on the presynaptic site; whereas if postsynaptic components are dysfunctional, animals show resistance to the levamisole [28]. Compared to the wild-type, *hlb-1 (ok725)* animals exhibited increased resistant to the levamisole (Fig. 4H). These results suggest that *hlb-1* may positively regulate aspects of synaptic transmission, and may be involved in the regulation of both the presynaptic differentiation and the postsynaptic differentiation.

3.7 Synaptic defects in *hlb-1* appear to function cell nonautonomously Because HLB-1 is predominantly expressed in muscle cells[12], we asked whether its expression was specifically required in neurons or muscles. Firstly, a series of transgenic lines were created containing genomic DNA fragments to rescue analysis by microinjection transformation. The response defects of *hlb-1* (*ok725*) mutants to the aldicarb and the *juIs1* marker defects could be rescued by pDY05 containing the full-length genomic DNA of *hlb-1*, but could not be restored by pDY02 without the C-terminal of *hlb-1* genomic DNA or pDY03 without the N-terminal of *hlb-1* genomic DNA (Fig. 5). Secondly, the genetic mosaic analysis was performed using *hlb-1(ok725); juIs1* animals carrying an extrachromosomal array containing a *hlb-1(+)* genome clone and a maker, SUR-5::GFP, which was expressed in the nuclei of all somatic cells[26]. Three animals that had lost the array from the muscle lineage were found to be wild-type after being scored for SNB-1::GFP puncta shape and distribution. Four animals that had lost the array from the neural lineage were also observed to have wild-type puncta. Therefore, the synaptic defects in *hlb-1 (ok725)* animals were rescued equally when the array was lost from the muscle lineage or neural lineage, demonstrating that HLB-1 functions cell nonautonomously.

3.8 *hlb-1* functions in parallel with *syd-2* to regulate the organization of neuromuscular junctions Data from Serra-Pagès *et al* (1998) demonstrated the physical interaction between C-terminal non-coiled coil regions of α–liprins and that of β–liprins[12], and the high homologue between HLB-1 and β–liprins, as well as that between SYD-2 and α–liprins[9] suggests the possible physical interaction between HLB-1 and SYD-2. The physical interaction between HLB-1 and SYD-2 has already been confirmed in *C. elegans* (D. Wang *et al*,

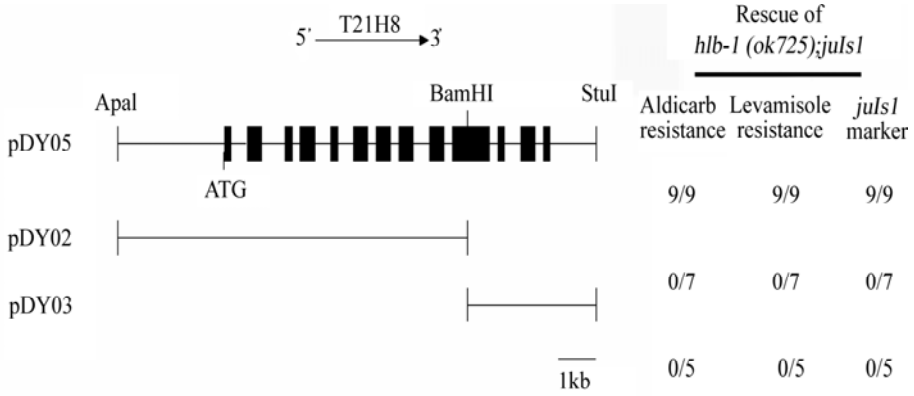

Fig. 5 ***hlb-1*** **constructs used to generate transgenic lines and rescue of** ***hlb-1 (ok725); juIs1*****. Rescue of** ***hlb-1 (ok725); juIs1*** **mutant phenotype is indicated on the right. Numbers indicate the number of rescued transgenic lines out of the total number of lines scored. The mutation phenotypes analyzed for the rescue experiment include defects of aldicarb resistance, levamisole resistance, and the** ***juIs1*** **fluorescent marker. Open boxes, exons.**

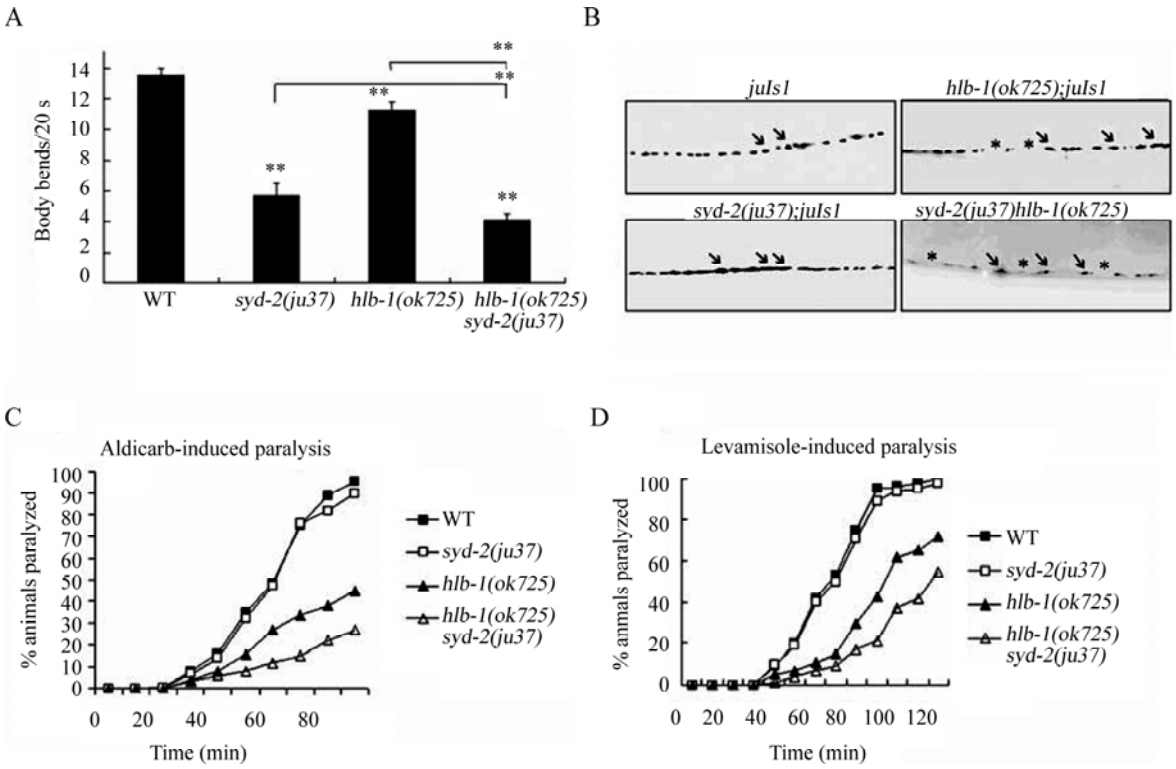

Fig. 6 Analysis of (A)body bends, (B)SNB-1::GFP puncta, (C)aldicarb-induced paralysis, (D)levamisole-induced paralysis in *hlb-1(ok725)syd-2(ju37)* double mutant animals, compared with wild-type or single mutant. The dorsal cords of GABAergic presynaptic terminals were visualized by *Punc-25*-SNB-1::GFP (*juIs1*). Arrows indicated the fluorescent puncta, and asterisks indicated the fluorescent gap. WT, wild-type N2. Error bars indicate SEM. **$P < 0.01$.

personal communication). Furthermore, to address the problem of whether *hlb-1* and *syd-2* function in the same or different genetic pathway to exert their different roles in regulating synaptic function during synaptogenesis, double mutant of *hlb-1*(*ok725*)*syd-2*(*ju37*) was constructed to examine the possible genetic interactions between *hlb-1* and *syd-2* in controlling the synaptic function. Firstly, the *hlb-1*(*ok725*)*syd-2*(*ju37*) double mutants showed more severe locomotion behavior defects than the single mutant of *hlb-1*(*ok725*) or *syd-2*(*ju37*) as revealed by the body bend assay (Fig. 6A). Secondly, we constructed the mutants of *hlb-1*(*ok725*)*syd-2*(*ju37*); *juIs1,* which possessed both the SNB-1::GFP phenotype of *syd-2*(*ju37*) and the fragment deletion in *hlb-1* (*ok725*) animals. We observed more severe SNB-1:: GFP puncta defects in *hlb-1*(*ok725*)*syd-2*(*ju37*) double mutants with noticeably decreased number of SNB-1::GFP puncta and markedly increased number of abnormal SNB-1::GFP puncta compared to *hlb-1*(*ok725*) mutant or *syd-2*(*ju37*) mutant (Fig. 6B). Thirdly, the response to aldicarb was tested in *hlb-1* (*ok725*)*syd-2*(*ju37*) double mutants, which exhibited more severe resistance to aldicarb than *hlb-1*(*ok725*) mutant or *syd-2*(*ju37*) mutant (Fig. 6C). Similarly, *hlb-1*(*ok725*)*syd-2* (*ju37*) double mutants also exhibited more severe resistance to levamisole than *hlb-1*(*ok725*) mutant or *syd-2*(*ju37*) mutant (Fig. 6D). These results demonstrate that *hlb-1* acts in a different and parallel pathway with *syd-2* to regulate the organization of neuromuscular junctions in *C. elegans*. That is, the complex, containing the HLB-1/ liprinβ1 and the SYD-2/ liprinα, mediates at least two different genetic pathways to control the active zone formation and the neurotransmission process, respectively.

3 Discussion

Although it has been proven that SYD-2 regulates the

active zone formation, the roles of HLB-1, another member of liprin family in *C. elegans*, in regulating the organization or function of neuromuscular junctions are still unclear so far. In the present study, we provide evidence to prove that HLB-1 may play an important role in regulating the organization and function of neuromuscular junctions in nematodes. Firstly, the deletion animals of *hlb-1 (ok725)* showed a significant impairment in the speed of straight stinusoidal locomotion as quantified by the body bends and a noticeably decreased frequency of thrashing for both the L1 larvae and adult nematodes compared to wild-type. Secondly, the *hlb-1 (ok725)* mutants showed abnormal SNB-1:: GFP puncta that were slightly diffused and usually more widely spaced. A decrease in the number of puncta in both ventral and dorsal cords was also found in *hlb-1(ok725)* mutants. Thirdly, percentage of abnormal SNB-1::GFP puncta in *hlb-1(ok725)* was about 7 folds as that in wild-type animals. Besides, the area of the presynaptic zone in *hlb-1(ok725)* was significantly enlarged compared to that in wild-type animals. The puncta of *hlb-1(ok725)* mutant appeared enlarged, diffused, and spaced farther apart than normal, and the area of the postsynaptic zone in *hlb-1(ok725)* was also significantly enlarged compared to that in wild-type animals. Further studies revealed that *hlb-1 (ok725*) animals were highly resistant to both aldicarb and levamisole. Thus, HLB-1 appeared as a new regulator for the organization and function of neuromuscular junctions in *C. elegans*.

In addition, our data also suggest that HLB-1 regulates the organization of neuromuscular junctions in a cell nonautonomously way, and HLB-1 functions differently from SYD-2 to regulate the synaptic functions in *C. elegans*. The expression patterns of active zone protein UNC-10 further verify that HLB-1 does not affect the presynaptic active zone morphology while SYD-2 does. Therefore, our data further suggest the possibility that *hlb-1* may play an important redundant role in addressing the *syd-2* functions at synaptic regions by regulating aspects of synaptic transmission. Liprin-α/SYD-2 is proposed to act as an intracellular anchor for RPTP signaling at synaptic junctions[9, 31], and it has been shown that Liprin-α/SYD-2 binds Rim, CAST/ERC and kinesin[10, 32-33]. In addition, for the possible functional link between SYD-2, LAR and Nidogen, reports indicated that SYD-2 might recruit other density components to facilitate its assemble, and the presynaptic activity of SYD-2 might be modulated by LAR-Nidofen interaction to secure the synaptic assembly[21,22]. HLB-1/ liprinβ1 motif also suggested that HLB-1/ liprinβ1 might encode another scaffold protein at synaptic since it had a similar SAM domain as that in SYD-2. Thus, liprinβ1/HLB-1 structure hints the possibility that HLB-1 may recruit components of extracellular matrix or postsynaptic receptors to regulate the organization of neuromuscular junctions. Moreover, the localization of HLB-1 in the same complex with SYD-2 further suggests the possible postsynaptic association of SYD-2 with HLB-1 in *C. elegans* as revealed in vertebrate synapses[34,35]. To target receptors, such as AMPA receptor, to the postsynaptic membrane, further studies are still required to completely elucidate the interaction between SYD-2 and HLB-1.

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HLB-1 参与秀丽线虫神经肌肉接头组装与功能的调控

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摘要：目的 研究基因 *hlb-1* 在秀丽线虫神经肌肉接头组装与功能调节中的作用。**方法** 通过 SNB-1::GFP 与 UNC-49::GFP 表达模式分析神经突触的结构变化，通过涕灭威与左旋四咪唑药物实验分析神经突触的功能变化，从而解析HLB-1在神经肌肉接头组装与功能调节中的作用。进一步通过恢复与嵌合分析实验，对HLB-1在神经肌肉接头组装与功能调节中的作用予以确认。**结果** 基因 *hlb-1* 功能的丧失会导致神经元生长缺陷和神经元的损失。*hlb-1* 的基因突变会引发SNB-1::GFP与UNC-49::GFP表达模式的改变，暗示前突触与后突触结构的改变。*hlb-1* 的基因突变可以引起明显的运动行为损伤，同时对涕灭威、左旋四咪唑的抗性也存在缺陷，表明 *hlb-1* 突变体中前突触与后突触功能的改变。恢复与嵌合分析实验表明 HLB-1 对于突触功能的调控是细胞非自主性的。而且，HLB-1 的表达并非前突触活动区形态建成所必需。遗传分析进一步指出 *hlb-1* 与 *syd-2* 作用于平行的通路上调控着神经突触的功能。**结论** HLB-1 功能上作为秀丽线虫神经肌肉接头组装与功能的新调节因子发挥作用。

关键词：HLB-1；突触功能；神经肌肉接头；SYD-2；秀丽线虫