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Hikaru genki protein is secreted into synaptic clefts from an early stage of synapse formation in *Drosophila*

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SUMMARY

The development of neural circuits is regulated by a large number of factors that are localized at distinct neural sites. We report here the localization of one of these factors, hikaru genki (hig) protein, at synaptic clefts in the pupal and adult nervous systems of *Drosophila*. In *hig* mutants, unusually frequent bursting activity of the muscles and abnormal motor behavior during the adult stage suggest the misfunction of neuromuscular circuitry. Our immunohistochemical analyses revealed that hig protein, produced by neurons, is secreted from the presynaptic terminals into the spaces between the presynaptic and postsynaptic terminals. In addition, we have found that the localization of this protein in the synaptic spaces temporally correlates with its functional requirement during a critical period that occurs in the middle stage of pupal formation, a period when a number of dendrite and axon growth cones meet to form synapses. These findings indicate that hig protein functions in the formation of functional neural circuits from the early stages of synapse formation.

Key words: *hikaru genki*, *Drosophila*, synaptogenesis, synapse, synaptic cleft, neural circuit

INTRODUCTION

The nervous system comprises a myriad of neurons that extend axons to make synaptic connections with appropriate target cells and that eventually form a highly complex neural network. This network provides the basis for a variety of neural functions, including the control of behavior, learning and memory. During the development of the nervous system, the following steps occur after the determination of neuronal identity: the outgrowth of axons and their guidance toward their destinations, the local selection of correct synaptic targets, and the formation and differentiation of synapses. In vertebrates and invertebrates, the analyses of these developmental steps have advanced from the descriptive to the molecular level, identifying an increasing number of relevant molecules (Goodman and Schatz, 1993; Hall and Sanes, 1993).

In *Drosophila*, several approaches have been taken in the study of the formation of the neural network, and extensive morphological and physiological analyses have been reported (Fernandes and Keshishian, 1995; Johansen et al., 1989; Broadie and Bate, 1993a,b; Kidokoro and Nishikawa, 1994). The small number of body wall muscles and motor neurons in the embryos and larvae of *Drosophila* have permitted the morphological analysis of how axons are guided to specific target muscles (Sink and Whitington, 1991; Keshishian et al., 1993). In addition, many molecules involved in axon guidance have been found in axon membranes and muscle fibers (for review, Fernandes and Keshishian, 1995). Three of those molecules, fasII, fasIII and connectin, induce errors in axon guidance or target recognition when ectopically expressed (Lin and Goodman, 1994; Chiba et al., 1995; Nose et al., 1994). Other factors involved in neural network formation have been studied using genetic approaches (Krishnan et al., 1993; Muralidhar and Thomas, 1993; Oh et al., 1994; Van Vactor et al., 1993). However, among these factors in *Drosophila*, no molecules localized in synaptic clefts have been identified.

In our previous study, we genetically screened *Drosophila* mutants that display abnormal motor activity in order to identify factors that contribute to the formation of neural circuits. When *hikaru genki* (*hig*), one of the factors identified, is affected, the mutant fly exhibits severely reduced or uncoordinated locomotion at the larval and adult stages. The *hig* gene encodes a protein that has sequences characteristic of secretory proteins, including an immunoglobulin (Ig) domain and three or four complement binding (CB) domains, all found in a variety of cell recognition molecules. In situ hybridization revealed that *hig* is specifically expressed in a subset of neurons in the CNS in late stage embryos (Hoshino et al., 1993). These behavioral and molecular data suggest that *hig* participates in some neuronal recognition events during the formation of functional neural circuits. In this paper, we report data obtained from electromusculography, immunohistochemistry and conditional rescue experiments that assess the role of hig protein. These data suggest that hig protein is secreted from the presynaptic terminals into the synaptic clefts and functions in the formation of functional neural circuits during synaptogenesis.

MATERIALS AND METHODS

Electromusculography

Adult flies, reared at 22ºC, were anesthetized with CO_2 for 2 minutes and then their whole bodies, including head, wings, legs and abdomen were fixed on a slide glass using heat-melted myristilic acid. A glass electrode filled with *Drosophila* Ringer solution was inserted into the dorsal longitudinal muscle (DLM) through the cuticle or, for reference, placed on the surface of the compound eye. The electrorecordings were performed at 22ºC under dark conditions. Each recording had a duration of 3 minutes.

Antibodies

Anti-hig antibody was raised against a fusion protein that consisted of the N-terminal portion of the hig protein (a.a.35 to 295) and a metal-binding domain derived from pTrcHis vector (Invitrogen Xpress System). Two oligonucleotides were designed as primers for a polymerase chain reaction that would amplify the DNA sequence encoding the N-terminal region, creating *Bam*HI and *Eco*RI sites at its ends. This DNA fragment was then ligated into pTrcHis A vector predigested with both *Bam*HI and *Eco*RI to generate the plasmid that produces the fusion protein. After the protein was overexpressed in the *E. coli* strain, TOP10, it was purified under denaturing conditions by immobilized metal affinity chromatography using Invitrogen's ProBond resin. The purified protein was run on a SDS-PAGE gel, excised and electroblotted onto a nitrocellulose filter. A filter containing 100 μg of the protein was fragmented and injected 3 to 4 times intraperitoneally into rats. After bleeding, antibody against hig protein was affinity-purified as previously described (Inuzuka et al., 1991).

Immunostaining

For whole-mount staining of adult brains and ventral ganglia, these organs were dissected from adult flies in PBS and fixed with 4% paraformaldehyde in PBS for one hour.

Sections of pupal and adult heads were prepared as follows. Pupal and adult heads were fixed in PLP fixative for 1.5 hours followed by successive incubations in 5% and 10% sucrose for 30 minutes at RT, 15% and 30% sucrose for one hour at RT, and 30% sucrose overnight at 4ºC. All sucrose solutions were in 0.1 M phosphate buffer (pH 7.0). After the heads were frozen in liquid N_2, 10 μm serial sections were cut with a cryostat microtome. They were washed three times with PBS for 5 minutes each, incubated in 0.15 M glycine in PBS for 15 minutes, and blocked with PBT'-SM (0.05% Triton X-100 in PBS with 5% skim milk) for light microscopy or with PBSS-SM (0.1% saponin in PBS with 5% skim milk) for electron microscopy. The blocked sections were incubated in the primary antibody solution (diluted 1:10 in PBT'-SM or PBSS-SM) at 4ºC overnight. After five washes with PBS, they were incubated with secondary antibody (biotin-conjugated anti-rat IgG, diluted 1:1000; Vector Labs) in PBT'-SM or PBSS-SM for one hour, again washed 5 times, incubated in the avidine-biotin complex solution (ABC Elite; Vector Labs) for one hour and washed five additional times. The samples for EM were fixed in 0.5% glutaraldehyde in PBS for 5 minutes followed by five washes with PBS before the addition of DAB solution (0.3 mg/ml diaminobenzidine, 0.00075% H_2O_2 in PBS).

Electron microscopic techniques

Thin sections (70 nm thick) for the electron microscopy illustrated in Fig. 6A,B were made as previously described (Matsumoto et al., 1988). For the immunoelectron microscopy, the specimens were processed using a standard technique (Suzuki and Hirosawa, 1994). For the samples in Fig. 6C,D, they were additionally incubated in 2% uranyl acetate solution for 5 seconds.

Rescue experiment

Plastic vials containing either larvae, pupae or adult flies were heat-shocked three times for 30 minutes each in a 37ºC water bath, with intervals of 30 minutes at 25ºC between heat shocks. The flies emerging from the pupal cases were collected at 12 hour intervals and subjected to a locomotor assay after 3 to 5 days. In this assay, approximately 20 flies were transferred at once to a plastic 50 ml graded cylinder that was divided into seven levels, each corresponding a measure from 0 to 6 from the bottom of the cylinder. After the flies were placed on the bottom of the cylinder, the number of flies able to reach each area in 15 seconds was determined. The scores were represented by the mean values of the points that individual flies obtained. This assay was repeated at least five times.

RESULTS

Defect in the motor circuitry of the *hig* null mutant

Flies homozygous for the *hig* null mutation (hig^{dd37}) cannot jump or fly; they can only walk slowly with an unstable gait. Furthermore, they sometimes exhibit body and wing tremors while either standing or walking. Since our previous study suggested that *hig* may participate in some neuronal recognition events, these behavioral phenotypes are likely to be caused by misfunctions of the neural circuits that affect muscle activity. Therefore, we examined the neural circuits of *hig* mutants by recording the spontaneous electrophysiological activity of the adult dorsal longitudinal muscles (DLMs). In this experiment, more than 60% of the null mutants examined showed unusually frequent bursting activity rarely observed in wild-type flies under our experimental conditions. This activity was detected simultaneously in muscles located at the two lateral sides (Fig. 1). These concurrent patterns cannot be explained by the spontaneous activity of distinct muscles and therefore were ascribed to the response of the muscles to motor nerve impulses. This indicates that there are defects in the

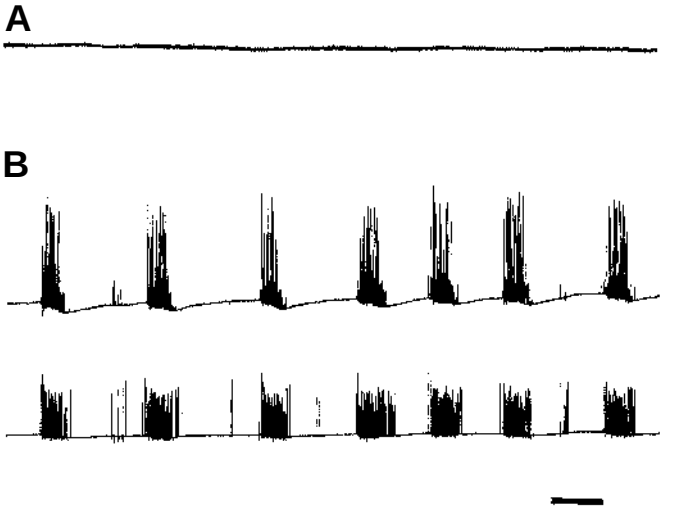

Fig. 1. Electromusculogram in the *hig* mutants. Spontaneous electrophysiological activities of the adult dorsal longitudinal muscles were recorded in the wild type (A) and in the *hig* null mutant, hig^{dd37} (B). In B, muscles located on both lateral sides of the thorax simultaneously displayed bursting activity. During a period from 10 hours to 4 days after eclosion, none of the wild-type flies (0 out of 21) showed bursting activity, whereas 16 out of 24 mutant flies exhibited extensive activity. The patterns shown here are typical for flies one day after eclosion. Bar, 10 seconds.

neural circuits of the CNS that simultaneously control the motoneurons involved in the activity of these muscles; we do not exclude the possibility that the mutant flies also have impaired neuromuscular junctions (NMJs). The evidence suggests that *hig* plays a crucial role in the formation of functional neural circuitry.

Localization of hig protein

To reveal the sites where *hig* functions in adult tissues, we used antibody raised against a hig fusion protein expressed in bacteria. This polyclonal antibody did not significantly stain the tissues of the *hig* null mutant (data not shown), indicating its specificity for hig protein. However, the hig antibody clearly stained, at various intensities, a large number of cells in the brain and ventral ganglion of wild-type flies. In the brain, the laminar cells in the optic lobe and some in the central brain showed strong staining (Fig. 2A). In the ventral ganglion, staining was observed, in a dotted pattern, along nerves that run on the surface of the ganglion (Fig. 2B) and that extend to the peripheral tissues. Staining was detected not only in the CNS, but also in the motor nerves and boutons of certain muscles (Fig. 2C), including DLMs. We could observe strong staining in the nerves of muscle 8 in the A2 and posterior abdominal segments of young adult flies. Other tissues, including muscles, did not show significant staining.

We further stained sections of the brain to examine the distribution of hig protein in its internal structures. The adult brain comprises two distinct regions, the cortical region and the neuropiles. The cortical region consists of neuronal cell bodies that extend their neurites inward and form a large number of synapses in the internal regions of the brain, the neuropiles. hig protein was found in both the neuropiles and cortical regions throughout the brain except for the retinular cells and the laminar neuropile (Fig. 2D). The individual neural cell bodies showed staining that excludes the nucleus.

To reveal the subcellular localization of hig protein, we performed immunoelectron microscopy. Antibody staining in the adult brain identified hig protein in the organelles involved in secretion in the neuronal soma - the endoplasmic reticulum/Golgi apparatus, vesicles and nuclear membrane

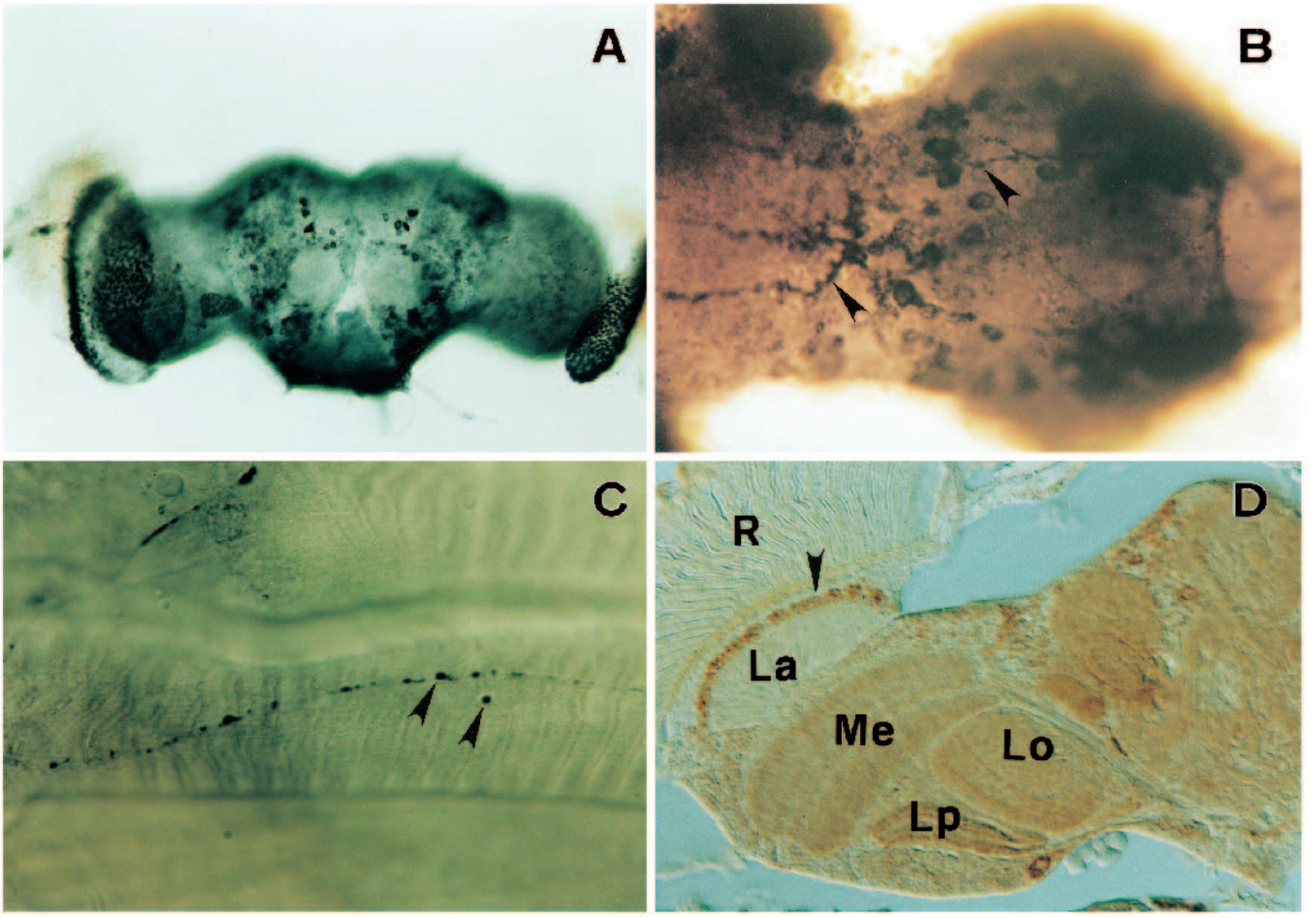

Fig. 2. Localization of hig protein in the adult CNS. (A) Whole-mount staining of adult brain. A number of cells show antibody staining with a variety of intensities. (B) Staining of adult ventral ganglion. The nerves that run on the surface of the ventral ganglion are stained in a dotted pattern (arrowheads). Note that staining of the cell bodies excludes the nuclei. Anterior is left. (C) Staining of the motor nerve. Boutons (arrowheads) of the motor nerves exhibit staining on a restricted number of muscles. (D) Cryosection of the adult visual system. Note the staining in the neuropiles of the optic ganglia (Me, Lo, Lp), except for the lamina (La). The lobula plate (Lp) shows the most prominent staining. A large number of cell bodies surrounding the neuropiles also show staining to various degrees. The cell bodies in the laminar layer (arrowhead) exhibit stronger signals. No expression was observed in the retinular cells (R) at this stage. Me, medulla; Lo, lobula.

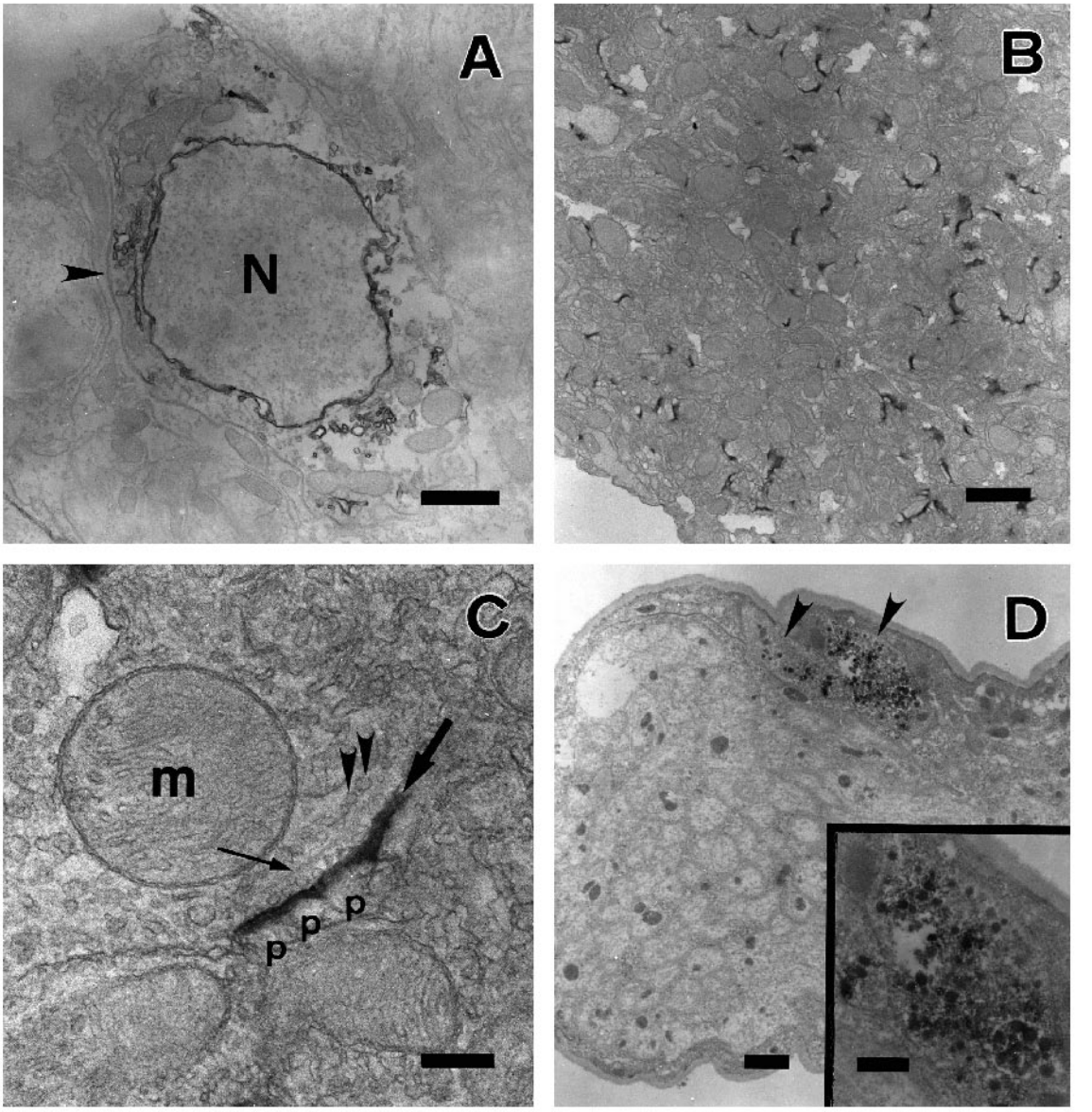

Fig. 3. Subcellular localization of hig protein using electron microscopy. (A) A laminar cell body. hig protein is localized in the endoplasmic reticulum/Golgi apparatus, nuclear membrane and vesicles. Note that there is no significant staining of the plasma membrane (arrowhead) of the cell body. N, nucleus. (B) Electron microscopy of the lobula plate in the adult brain. hig staining is observed in a number of synaptic clefts. (C) Higher magnification of the lobula plate. A synaptic cleft (thick arrow) is specifically stained with hig antibody. The presynaptic structure can be discriminated by the synaptic vesicles (arrowheads), platform of the synaptic ribbon (thin arrow), and mitochondria (m). The postsynapses (p) are small and round. (D) A cross section of the peripheral nerve extending from the adult thoracic ganglion. Two neural processes in the bundle contain clusters of vesicles showing hig staining (arrowheads). The inset shows the vesicles, at a higher magnification, many of which are stained. Scale bars, 1 μm in A, B, and D; 500 nm in D (inset); 200 nm in C.

(Fig. 3A). The most striking observation is that, in the neuropiles of the adult brain, large quantities of hig protein were localized in a number of discrete intercellular spaces bordered by cell membranes (Fig. 3B).

The structure of the synapse of the *Drosophila* brain has several morphological characteristics (see Fig. 6A). The presynaptic terminal is enlarged and contains a number of synaptic vesicles concentrated in active zones which are occasionally marked by electron-dense synaptic ribbons. This terminal, in many cases, faces a cluster of several small postsynaptic terminals, and the spaces between them are the synaptic clefts, which are wider than other intercellular spaces. The surfaces of both the pre- and postsynaptic terminals are electron-dense. These morphological properties have been similarly observed in the synapses of the vertebrate brain. We observed that the spaces labeled with hig staining were clearly wider than other intercellular regions and were surrounded by the structures characteristic of pre- and postsynaptic terminals (Fig. 3C). We therefore conclude that the intercellular spaces where hig protein accumulates are synaptic clefts.

We could not find hig protein along transport pathways of the neuropiles of the adult CNS, but it was detected in the vesicles of varicosities of a small number of neural processes in the peripheral nerves (Fig. 3D). These neural processes,

filled with the vesicles, possibly stem from neurons or neurosecretory cells. They are unlikely to arise from glia since light microscopy showed hig staining along the peripheral nerves only in a few contiguous rows accompanying a dotted pattern of varicosities. These staining patterns found in several neural tissues outline a possible pathway of hig protein transport: it is synthesized in the neuronal soma, transported through the axons in vesicles and then secreted from the presynaptic terminals into the synaptic clefts.

Requirement of hig protein during the middle pupal stage for normal adult locomotion

We previously reported that the *hig* behavioral phenotype could be rescued by the introduction of a *hig* mini-gene into the *hig^{dd37}* null mutant flies (Hoshino et al., 1993). One transgenic line, *hsD12-87*, expresses *hig* conditionally after heat shock and was used to determine when *hig* is required for the normal locomotor activity of adult flies. Initially, we heat-shocked *hig^{dd37}* flies carrying *hs-hig* once a day throughout their development and found that only heat shock during pupariation rescued adult locomotion. We then focused on the analyses of the pupa and adult. When control *hig^{dd37}* flies were heat-shocked at any stage of their development, their motor behavior could not be rescued. In contrast, mutant flies that expressed *hs-hig* during the middle of their pupal stage were found to behave normally in adulthood (the critical period is 72-24 hours before eclosion, Fig. 4). They were able to jump and fly, and did not exhibit body tremors. Expression of *hs-hig* at other developmental stages conferred little or no rescue effect. We observed that the heat shock of both *hig^{dd37}* with and without *hs-hig* at the −12 to 0 hour point of the pupal stage caused their untimely death in the pupal cases, and heat shock after eclosion resulted in paralysis within a few minutes followed by death within 10 hours. However, the locomotor behavior and viability of the *hig^{dd37}* mutants that expressed *hs-hig* during the critical period were not affected by additional heat shock during the late pupal or adult stages; wild-type flies are also unaffected by these latter-stage heat shocks. Therefore, *hig* expression in the critical period also rescues the heat-shock susceptibility of mutant flies at −12 to 0 hours and after eclosion. These results indicate that hig protein is developmentally required at particular stages during pupariation for the formation of normal neural circuitry.

Transport and localization of hig protein during pupal development

We subsequently examined hig protein localization in wild-type animals during pupal stages, especially in the critical period identified by the rescue experiments. hig protein was initially localized predominantly in the cell bodies of the young pupal brain, but in pupae during the critical period, hig protein accumulated in the neuropiles of all brain regions and only in a fraction of the cell bodies (Fig. 5A). At the later pupal stages, hig protein disappeared from the neuropiles and was again observed predominantly in the neuronal soma (Fig. 5B). Therefore, hig protein that appears in the neuropiles during the critical period does not persist in later pupal stages. However, in the adult brains, hig protein again accumulated in the neuropiles and in the soma (see Fig. 2D).

The distribution patterns of heat-induced hig protein were also examined in *hig^{dd37}* transgenic flies to monitor its dynamic

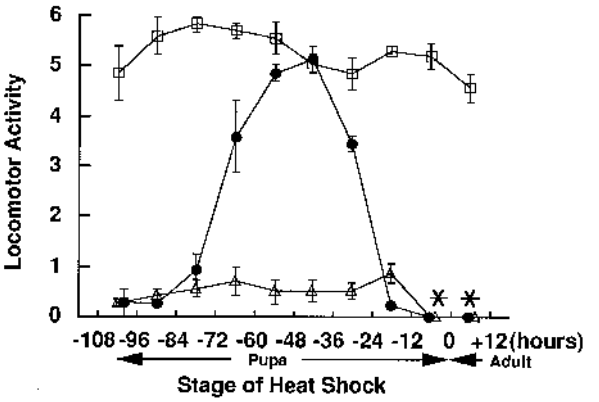

Fig. 4. Requirement of hig protein during pupariation for normal adult locomotion. *yw* (wild type, open square), *yw*;*hig^{dd37}*(*hig* null allele, open triangle), and *yw*; *hig^{dd37}*;*hsD12-87*/+ (closed circle) were heat-shocked at the developmental time indicated and their locomotor activity at the adult stage was plotted. The time is shown in hours until or after eclosion. Heat shocks of *yw*; *hig^{dd37}* and *yw*; *hig^{dd37}*; *hsD12-87*/+ at −12 to 0 hours caused death, and heat shocks at the adult stage resulted in death within 10 hours after the treatment (shown by asterisks). Mutant flies once rescued by the induction of *hig* during the critical period were not affected by additional heat shocks at later stages. Locomotor activity of flies that were not heat shocked is essentially the same as that of each strain heat-shocked at −84 hours. Similar results, including the stage-dependent recovery of adult locomotion, were also observed for the hypomorphic allele, *hig^{P1}* (data not shown). The error bars represent standard deviation.

movement. When hig protein was transiently heat-induced during the critical period, staining was detected in the neuropiles as well as in the chiasma regions of the axon tracts, but not in the cell bodies as measured 2.5 hours after the end of induction (Fig. 5C). This indicates that hig protein is immediately transported toward the axon terminals at this stage. However, when hig protein was induced at later pupal stages, most staining remained in the cell bodies even 5 hours after the onset of heat shock, indicating that the transport of hig protein is inhibited at these stages (Fig. 5D). These observations show that there is a stage-dependent regulation of hig protein transport. This regulation explains the patterns of the endogenous hig protein distribution in the wild-type pupal brain and actively localizes hig protein in the neuropiles when it is required. Thus, these findings suggest that hig protein functions in the neuropiles during the critical period for the development of the neural circuits.

hig protein appears in the synapses during the critical period

Since the neuropiles of the adult CNS contain a great number of synapses, we were interested in studying the developmental state of synapse formation during the critical period. We then examined the pupal CNS from this period using electron microscopy (EM). In contrast to the neuropiles of the adult brain that are densely populated with synaptic terminals of mature structure (e.g. electron-dense cell membranes, synaptic vesicles, etc.; Fig. 6A), the neuropiles of the pupal CNS of the critical period was rather sparsely populated with growth cones even in its most densely packed regions, and most of them had an unspecialized, rod-like appearance (Fig. 6B). Nevertheless,

some growth cones were seen to be in contact and their terminals were marked by electron-dense materials. This indicates that the growth cones had started to differentiate into synapses. These observations reveal that the critical period corresponds to an early stage of synapse formation.

Using EM, we further examined the localization of hig protein in the neuropiles of the critical period to identify the sites of hig action at a subcellular level. hig staining was occasionally observed inside the terminals, showing that hig protein has not yet been secreted from some terminals at this stage. However, in most cases, hig protein was found in the clefts between the closely positioned growth cones (Fig. 6C). In addition, some presynaptic growth cones extended several filopodia-like structures that appeared to be in contact with postsynaptic components and had already secreted hig protein at the multiple contact sites (Fig. 6D). We did not find staining outside growth cone membranes that had not yet contacted other growth cones. These observations indicate that, during the critical period, hig protein accumulates in the spaces where growth cones meet to form synapses, and therefore suggest that hig protein may be involved in synapse formation from its early stage after contact between synaptic pairs has been made.

DISCUSSION

hig is required for the development of functional neural circuits

To identify a factor involved in neural circuit formation, we previously isolated *hig* mutant flies based on their abnormal motor behavior (Hoshino et al., 1993). The mutant flies were found to exhibit occasional seizure-like behavior and heat-induced paralysis followed by death. These mutant phenotypes, together with the localization of hig protein in the nervous system, suggest that there are defects in the neural circuits of the *hig* mutant. More direct evidence was provided by recording the spontaneous muscular activity of the mutant flies. Abnormally frequent bursting activity simultaneously detected in different muscles indicates that the defect occurs in the part of the CNS that governs the neuromuscular circuitry. This misfunction of the CNS can be caused by either abnormal development of the neural network or impairment of a mechanism required for the neural function, such as neurotransmission. The developmental profile of *hig* transcripts, which are expressed both in the developing and the adult nervous system (Hoshino et al., 1993), did not discriminate these possible defects. To determine the role of *hig* in the generation of normal motor behavior, we performed conditional rescue experiments of *hig* mutants in which *hig* expression was transiently heat-induced throughout their development. These experiments identified a critical period during which *hig* is required for normal adult circuit formation. Since the critical period occurs during the middle pupal stage and hig protein synthesized in this period does not persist in the neuropiles at later pupal stages, *hig* must be involved in a developmental process. We therefore conclude that hig protein plays a crucial role for the development of normal neural circuits.

Localization of hig protein in the synaptic clefts

Our immunohistochemical analyses showed that hig protein is specifically localized in certain areas of the nervous system at pupal and adult stages. It was observed in neuronal cell bodies and axons, and in the neuropiles of pupal and adult brains. EM analyses revealed how hig protein is distributed in these subcellular regions. It was detected in the organelles constituting the secretory pathways of the cell body, and in the vesicles of the adult peripheral nerves. In the neuropiles of the adult brain, hig protein had a strikingly considerable presence in the

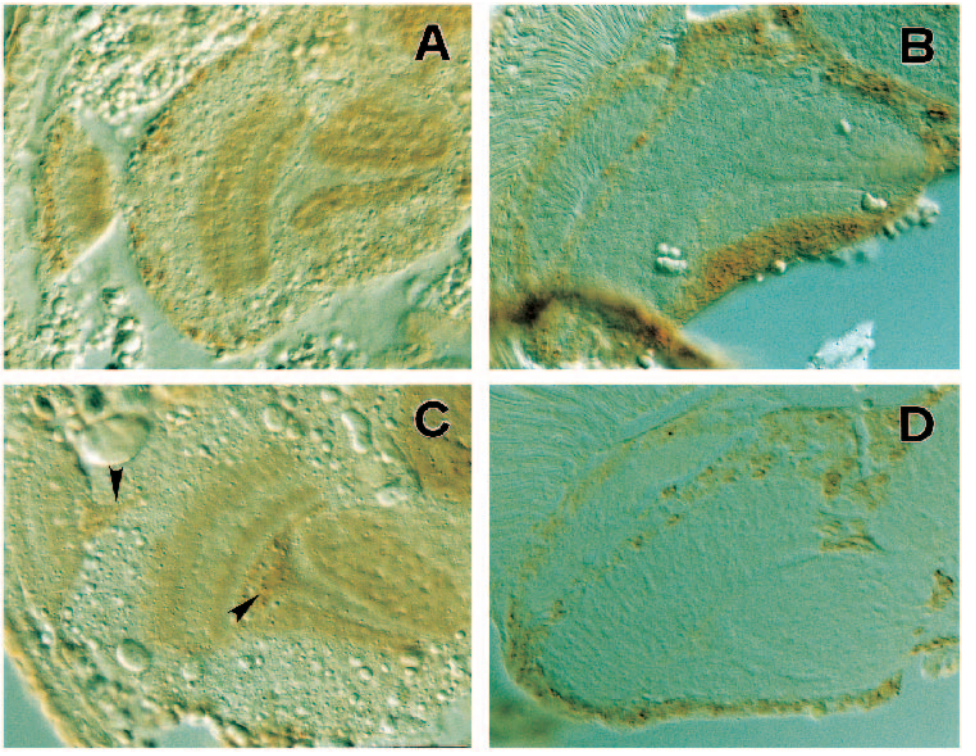

Fig. 5. Dynamic changes of hig protein localization during pupariation. (A,B) Distribution of hig protein in the optic lobe of wild-type pupa. At −55 to −50 hours (A), staining was observed in all the neuropiles of each optic ganglion. Note the localization of hig protein in the lamina which does not stain at the adult stage. However, at −20 to −10 hours (B), the signals in the neuropiles were extinguished, but appeared in regions of cell body. (C,D) Distribution of hig protein in the optic lobe of *yw*; hig^{dd37};*hsD12-87*/+ pupa heat-shocked at −57 to −49 hours (C) and at −15 to −5 hours (D). Transport of the heat-induced hig protein in the null mutant was regulated similarly to the normal hig protein of wild-type flies at the same stages. Note that the induced protein is observed in the axons in the regions of the chiasma (arrowheads) in C. For detection of the induced protein with antibody, the brains were fixed 2.5 hours after heat shock as described in Materials and Methods. hig protein induced at −55 to −50 hours did not persist until the adult stage.

synaptic clefts. These observations imply that hig protein is synthesized in the neuronal soma, transported through axons in vesicles and secreted from presynaptic terminals into the synaptic clefts.

The secretory nature of hig protein is consistent with its predicted structure, which includes a signal sequence but no transmembrane region. hig protein also contains an immunoglobulin (Ig) and three or four complement binding (CB) domains (Hoshino et al., 1993). In vertebrates and invertebrates, many molecules of the Ig superfamily are expressed in the developing nervous system and involved in neural network formation through certain recognition events (for reviews, Hynes and Lander, 1992; Horsch and Goodman, 1991). The CB domains are also found in proteins that participate in cell recognition (Hynes et al., 1992). These comparisons suggest that hig protein is one of the neural recognition molecules that function in the formation of the neural network. Among the recognition molecules in *Drosophila*, fasII, fasIII (reviewed in Hortsch and Goodman, 1991) and neuromusculin (Kania et al., 1993) belong to the Ig superfamily and are known to participate in axonal pathfinding and fasciculation (Grenningloh et al., 1991; Lin et al., 1994; Patel et al., 1987; Chiba et al., 1995). These molecules are localized on the axon membrane, as shown by contiguous antibody staining patterns under the light or electron microscope (Lin et al., 1994). In contrast, hig protein is found along axons in a dotted pattern, typically observed in the ventral ganglion. This protein distribution is rather similar to the staining patterns of molecules transported in vesicles, such as neuropeptides (Anderson et al., 1988; Cantera and Nässel, 1992; Gorczyca et al., 1993). Our EM analyses showed that hig protein is not localized on the axon membrane but in the pathways for vesicle secretion and in the synaptic clefts. These observations highlight a distinct role of hig protein compared with the known molecules participating in neural network formation and raise the possibility that hig protein functions in the synaptic clefts.

Our analyses further revealed that the subcellular localization of hig protein dynamically changes in a stage-dependent manner and that this protein is actively transported toward synaptic terminals during the period when it is functionally required. In the neuropiles of this critical period, some growth cones make contact with each other and exhibit features of early developing synapses. This indicates that the critical period corresponds to the early stage of synapse formation. Our immunohistochemical analyses showed that the immature presynaptic growth cones have already secreted hig protein into the spaces between the synaptic terminals at this stage. In addition, it should be noted that hig protein was not found outside the membranes of growth cones that had not yet made pairs. On the basis of these

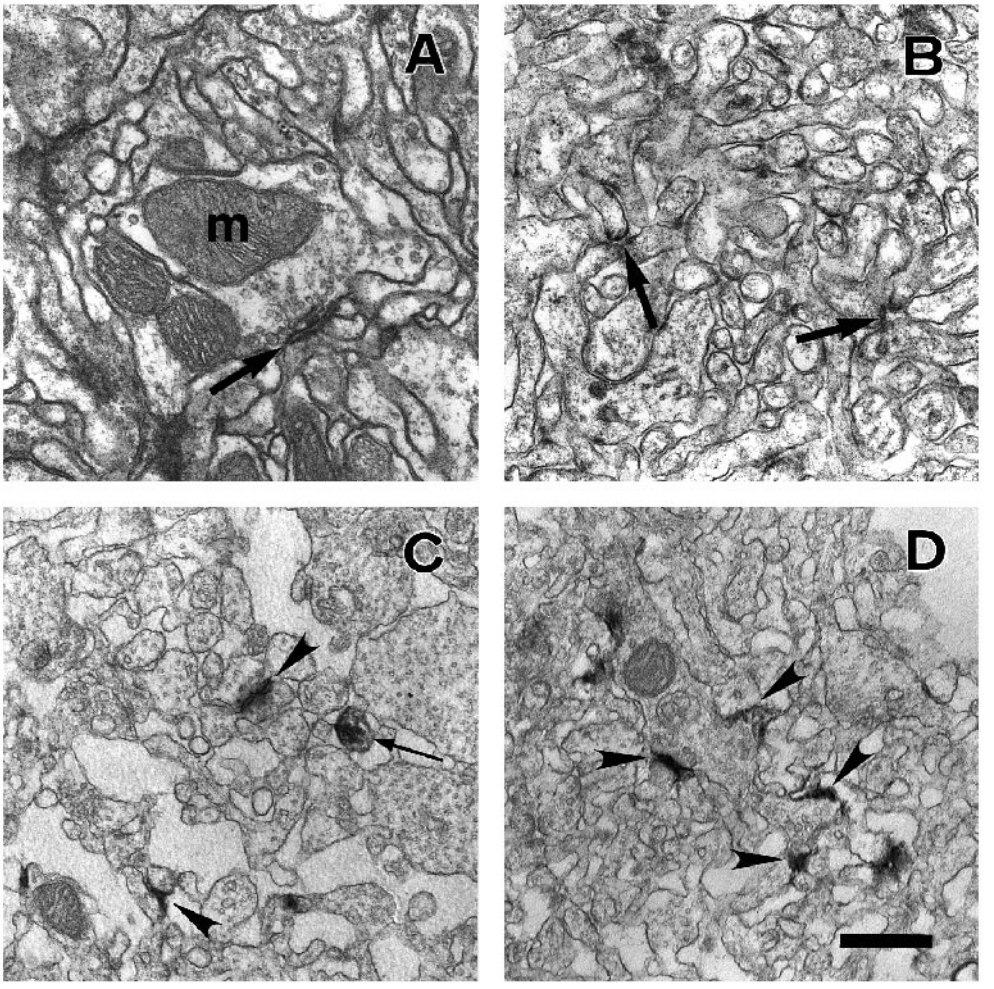

Fig. 6. The structure and hig staining of the synaptic contact sites during the critical period. (A) Morphology of a synapse of the adult brain. Both axons and dendrites are densely packed and exhibit the specialization of mature synapses: clusters of synaptic vesicles in large presynaptic terminals, active zones with an electron-dense synaptic ribbon, synaptic clefts having slightly wider contiguous spaces (arrows) surrounded by electron-dense membranes and small multiple postsynaptic terminals. Similar structures were observed throughout the adult CNS. m, mitochondrion. (B) Medulla of the pupal brain at –67 to –62 hours. Density of the growth cones or synaptic structures varies in different regions of the CNS at this stage. This picture shows an area of the most dense region. Note that most synaptic terminals marked with electron-dense patches (arrows) are small and untightly packed, showing immature synapse formation. Regions populated by mature synapses were rarely found at this stage. (C) hig protein at the sites of synaptic contact in the medulla of the pupal brain at–67 to –62 hours. hig staining was observed in the spaces between the opposed faces of growth cones (arrowheads). One growth cone appears to still contain hig protein inside its terminal (thin arrow). (D) An axonal growth cone exhibits several filopodia-like extensions that contact surrounding neurites. hig protein accumulates at the multiple contact sites (arrowheads). The extensions include clusters of vesicles, indicating that they are presynaptic components. Scale bar, 500 nm.

data, we propose that hig protein functions in synaptic clefts from an early stage of synapse formation after the growth cones have made contact with each other.

We have described the requirement of hig function during the critical period of the pupal stage. However, a large amount of hig protein again appears in the neuropiles of the adult CNS, although it is not apparently required for adult locomotion. This suggests another role for hig protein at the adult stage. We cannot presently assess its role in the adult CNS, but it is possible that a similar function might be at play in postdevelopmental events such as the maintenance of synapses or synaptic plasticity.

A possible role of hig protein in synapse formation

The distribution patterns of hig protein suggest that only subsets of synapses are affected in the mutant. For example, *hig* is expressed in 10% of neurons in the embryonic CNS at stage 17 (Hoshino et al., 1993) and hig protein is only observed in a small proportion of the NMJs of muscle 8 of third instar larva (unpublished data) and of a restricted number of adult muscles. It is therefore possible that, in certain areas of the mutant, both the normal and disrupted synapses are in close proximity, and that the normal synapses may mask the mutant phenotypes. This may partly explain why no unequivocal alterations have been detected in the synaptic morphology of the adult CNS and in the electrophysiological properties of the NMJs of muscle 8 of third instar larvae (Ueda, Hama, and Kidokoro; unpublished data). Nevertheless, the data of our mutant and immunohistochemical analyses suggest that some synapses display altered phenotypes in the mutant. More detailed examination of the mutant nervous system may reveal subtle or regional alterations in synaptic properties that will clarify the function of hig protein.

Previous studies indicate that synaptogenesis is a lengthy process that involves an as yet undetermined number and variety of molecules in the spaces between the synaptic terminals. Among the molecules that have been analyzed to date are agrin and neuregulin, both of which accumulate in the clefts of the NMJ and induce aggregation or expression of acetylcholine receptors (McMahan, 1990; Rupp et al., 1991; Smith et al., 1992; Tsim et al., 1992; Falls et al., 1993; Jo et al., 1995). These molecules and hig protein share several properties. For example, they are transported through axons and secreted into the synaptic clefts from the presynaptic terminals during synaptogenesis. In addition, they consist of domains that are known to interact with other molecules. We therefore postulate that hig protein may provide a signal from the presynaptic neuron to form proper connections with the postsynaptic cell in the synaptic cleft. It is also noteworthy that we have found that hig protein is localized in the synaptic clefts of neuron-neuron synapses in the CNS because, to date, most molecules specifically localized in the clefts have only been found in the NMJ. The study of hig protein may provide clues to the understanding of how synapses develop a variety of properties in the organization of functional neural circuits in the complex nervous system.

Conclusions

Our data from antibody staining indicates that hig protein is secreted into the synaptic cleft from the presynaptic terminal. Furthermore, a rescue experiment identified the critical period when hig function is transiently required for the formation of adult motor circuitry. During this period, a number of growth cones of axons and dendrites are in the early process of differentiation of synapses, and hig protein is localized in the nascent synaptic clefts. The loss of the protein causes abnormal circuitry in the CNS as revealed by the altered behavioral phenotypes and electrical bursting activity in the mutant flies. These data indicate that hig protein, localized in synaptic clefts, contributes to the formation of functional neural circuits from the early stage of synapse formation.

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REFERENCES

Anderson, M. S., Halpern, M. E. and Keshishian, H. (1988). Identification of the neuropeptide transmitter proctolin in Drosophila larvae: characterization of muscle fiber-specific neuromuscular endings. *J. Neurosci.* **8**, 242-255.

Broadie, K. S. and Bate, M. (1993a). Development of the embryonic neuromuscular synapse of Drosophila melanogaster. *J. Neurosci.* **13**, 144-166.

Broadie, K. S. and Bate, M. (1993b). Development of larval muscle properties in the embryonic myotubes of Drosophila melanogaster. *J. Neurosci.* **13**, 167-180.

Cantera, R. and Nässel, D. R. (1992). Segmental peptidergic innervation of abdominal targets in larval and adult dipteran insects revealed with an antiserum against leucokinin I. *Cell Tissue Research* **269**, 459-471.

Chiba, A., Snow, P., Keshishian, H. and Hotta, Y. (1995). Fasciclin III as a synaptic target recognition molecule in Drosophila. *Nature* **374**, 166-168.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S. and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell* **72**, 801-815.

Fernandes, J. and Keshishian, H. (1995). Neuromuscular development in Drosophila: insights from embryos and pupae. *Current Opinion in Neurobiology* **5**, 10-18.

Goodman, C. S. and Shatz, C. J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell/Neuron* **72/10 (Suppl.)**, 77-98.

Gorczyca, M., Augart, C. and Budnik, V. (1993). Insulin-like receptor and insulin-like peptide are localized at neuromuscular junctions in Drosophila. *J. Neurosci.* **13**, 3692-3704.

Grenningloh, G., Rehm, E. J. and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in *Drosophila*: Fasciclin II functions as a neuronal recognition molecule. *Cell* **67**, 45-57.

Hall, Z. W. and Sanes, J. R. (1993). Synaptic structure and development: the neuromuscular junction. *Cell/Neuron* **72/10 (Suppl.)**, 99-121.

Hortsch, M. and Goodman, C. S. (1991). Cell and substrate adhesion molecules in *Drosophila*. *Annu. Rev. Cell Biol.* **7**, 505-557.

Hoshino, M., Matsuzaki, F., Nabeshima, Y. and Hama, C. (1993). *hikaru genki*, a CNS-specific gene identified by abnormal locomotion in Drosophila, encodes a novel type of protein. *Neuron* **10**, 395-407.

Hynes, R. O. and Lander, A. D. (1992). Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell* **68**, 303-322.

Inuzuka, H., Miyatani, S. and Takeichi, M. (1991). R-cadherin: a novel Ca^{2+}-dependent cell-cell adhesion molecule expressed in the retina. *Neuron* **7**, 69-79.

Jo, S. A., Zhu, X., Marchionni, M. A. and Burden, S. J. (1995). Neuregulins are concentrated at nerve-muscle synapses and activate ACh-receptor gene expression. *Nature* **373**, 158-161.

Johansen, J., Halpern, M. E. and Keshishian, H. (1989). Axonal guidance and the development of muscle fiber-specific innervation in Drosophila embryos. *J. Neurosci.* **9**, 4318-4332.

Kania, A., Han, P.-L., Kim, Y.-T. and Bellen, H. (1993). *neuromusculin*, a Drosophila gene expressed in peripheral neuronal precursors and muscles, encodes a cell adhesion molecule. *Neuron* **11**, 673-687.

Keshishian, H., Chiba, A., Chang, T. N., Halfon, M. S., Harkins, E. W., Jarecki, J., Wang, L., Anderson, M., Cash, S., Halpern, M. E. and Johansen, J. (1993). Cellular mechanisms governing synaptic development in Drosophila melanogaster. *J. Neurobiol.* **24**, 757-787.

Kidokoro, Y. and Nishikawa, K. (1994). Miniature endplate currents at the newly formed neuromuscular junction in Drosophila embryos and larvae. *Neurosci. Res.* **19**, 143-154.

Krishnan, S. N., Frei, E., Swain, G. P. and Wyman, R. J. (1993). *Passover*: a gene required for synaptic connectivity in the giant fiber system of Drosophila. *Cell* **73**, 967-977.

Lin, D. M. and Goodman, C. S. (1994). Ectopic and increased expression of fasciclin II alters motoneuron growth cone guidance. *Neuron* **13**, 507-523.

Lin, D. M., Fetter, R. D., Kopczynski, C., Greningloh, G. and Goodman, C. S. (1994). Genetic analysis of fasciclin II in Drosophila: defasciculation, refasciculation, and altered fasciculation. *Neuron* **13**, 1055-1069.

Matsumoto, E., Hirosawa, K., Takagawa, K. and Hotta, Y. (1988). Structure of retinular cells in a Drosophila melanogaster visual mutant, *rdg A*, at early stages of degeneration. *Cell Tissue Res.* **252**, 293-300.

McMahan, U. J. (1990). The agrin hypothesis. *Cold Spring Harbor Symp. Quant. Biol.* **55**, 407-418.

Muralidhar, M. G. and Thomas, J. B. (1993). The Drosophila *bendless* gene encodes a neural protein related to ubiquitin-conjugating enzymes. *Neuron* **11**, 253-266.

Nose, A., Takeichi, M. and Goodman, C. S. (1994). Ectopic expression of connectin reveals a repulsive function during growth cone guidance and synapse formation. *Neuron* **13**, 525-539.

Oh, C. E., McMahon, R., Benzer, S. and Tanouye, M. A. (1994). *bendless*, a Drosophila gene affecting neuronal connectivity, encodes a ubiquitin-conjugating enzyme homolog. *J. Neurosci.* **14**, 3166-3179.

Patel, N. H., Snow, P. M. and Goodman, C. S. (1987). Characterization and cloning of fasciclin III: A glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* **48**, 975-988.

Rupp, F., Payan, D. G., Magill-Solc, C., Cowan, D. M. and Scheller, R. H. (1991). Structure and expression of a rat agrin. *Neuron* **6**, 811-823.

Sink, H. and Whitington, P. M. (1991). Pathfinding in the central nervous system and periphery by identified embryonic Drosophila motor axons. *Development* **112**, 307-316.

Smith, M. A., Magill-Solc, C., Rupp, F., Yao, M. Y.-M., Schilling, J. W., Snow, P. and McMahan, U. J. (1992). Isolation and characterization of an agrin homolog in the marine ray. *Mol. Cell Neurosci.* **3**, 406-417.

Suzuki, E. and Hirosawa, K. (1994). Immunolocalization of a Drosophila phosphatidylinositol transfer protein (rdgB) in normal and rdgA mutant photoreceptor cells with special reference to the subrhabdomeric cisternae. *J. Electron Microsc.* **43**, 183-189.

Tsim, K. W. K., Ruegg, M. A., Escher, G., Kröger, S. and McMahan, U. J. (1992). cDNA that encodes active agrin. *Neuron* **8**, 677-689.

Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R. and Goodman, C. S. (1993). Genes that control neuromuscular specificity in Drosophila. *Cell* **73**, 1137-1153.

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