

# The Matrix Protein Hikaru genki Localizes to Cholinergic Synaptic Clefts and Regulates Postsynaptic Organization in the *Drosophila* Brain

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The synaptic cleft, a crucial space involved in neurotransmission, is filled with extracellular matrix that serves as a scaffold for synaptic differentiation. However, little is known about the proteins present in the matrix and their functions in synaptogenesis, especially in the CNS. Here, we report that Hikaru genki (Hig), a secreted protein with an Ig motif and complement control protein domains, localizes specifically to the synaptic clefts of cholinergic synapses in the *Drosophila* CNS. The data indicate that this specific localization is achieved by capture of secreted Hig in synaptic clefts, even when it is ectopically expressed in glia. In the absence of Hig, the cytoskeletal scaffold protein DLG accumulated abnormally in cholinergic postsynapses, and the synaptic distribution of acetylcholine receptor (AChR) subunits  $D\alpha 6$  and  $D\alpha 7$  significantly decreased. *hig* mutant flies consistently exhibited resistance to the AChR agonist spinosad, which causes lethality by specifically activating the  $D\alpha 6$  subunit, suggesting that loss of Hig compromises the cholinergic synaptic activity mediated by  $D\alpha 6$ . These results indicate that Hig is a specific component of the synaptic cleft matrix of cholinergic synapses and regulates their postsynaptic organization in the CNS.

**Key words:** acetylcholine receptor; DLG; *Drosophila*; matrix; synaptic cleft; synaptogenesis

## Introduction

In both vertebrates and invertebrates, proteins secreted to the extracellular synaptic spaces regulate clustering of neurotransmitter receptors (Broadie et al., 2011; Nakayama and Hama, 2011). For example, Agrin in vertebrates (Wu et al., 2010) and LEV-9 and OIG-4 in *Caenorhabditis elegans* (Gendrel et al., 2009; Rapti et al., 2011) induce clustering of AChR at cholinergic neuromuscular junctions (NMJs). In the mouse cerebellum, Cbln1, which links Neurexin to the glutamate receptor GluD2 at synapses (Matsuda et al., 2010; Uemura et al., 2010), is capable of prompting GluD2 clustering in heterologous cells. In *Drosophila* glutamatergic NMJs, clustering of glutamate receptors depends on the secreted protein Mind-the-Gap (Rohrbough et al., 2007). Although these secreted proteins have been identified in a few specific types of synapses, the identities of the matrix proteins that regulate the organization of cholinergic synapses in the CNS remain unknown.

The *hikaru genki* (*hig*) gene was identified by analysis of *Drosophila* mutants exhibiting reduced locomotor behavior. This gene is expressed in a subset of neurons in the CNS of embryos and continues to be expressed in the nervous system until the adult stage. Hig is a secretory protein with an immunoglobulin (Ig) domain and a maximum of five complement control protein (CCP) domains (Hoshino et al., 1993). Immunoelectron microscopy reveals that Hig localizes to the synaptic clefts of subsets of synapses in the adult brain and in nascent synapses during pupal stages (Hoshino et al., 1996). This protein is present in the secretory apparatus (Hoshino et al., 1996) and is secreted from cultured cells to the medium (Hoshino et al., 1999). Mutant phenotypes of *hig* flies can be rescued by transient expression of Hig during middle pupal stages, suggesting that Hig plays a role in differentiation of synapses (Hoshino et al., 1996).

Here, we show that Hig is a matrix protein localized at the synaptic clefts of cholinergic synapses in the CNS and regulates the distribution of AChR subunits and DLG in postsynaptic terminals. This study indicates a possible avenue for revealing the composition of synaptic cleft matrix and determining how the matrix is involved in differentiation of cholinergic synapses in the CNS.

## Materials and Methods

*Drosophila* strains. *hig*<sup>Δ37</sup> flies harbor a null mutation caused by a deficiency in the *hig* gene (Hoshino et al., 1993). The mutant strain *nAcRα-30D<sup>DAS1</sup>* (hereafter *Dα6<sup>DAS1</sup>*) (Watson et al., 2010) was obtained from the Bloomington Stock Center. *Dα7<sup>ΔEY6</sup>*, *Dα7-GAL4*, and *UAS-Dα7-EGFP* were obtained from H.J. Bellen (Fayyazuddin et al., 2006). To generate *UAS-hig-GFP*, *hig-GFP* was amplified by PCR from

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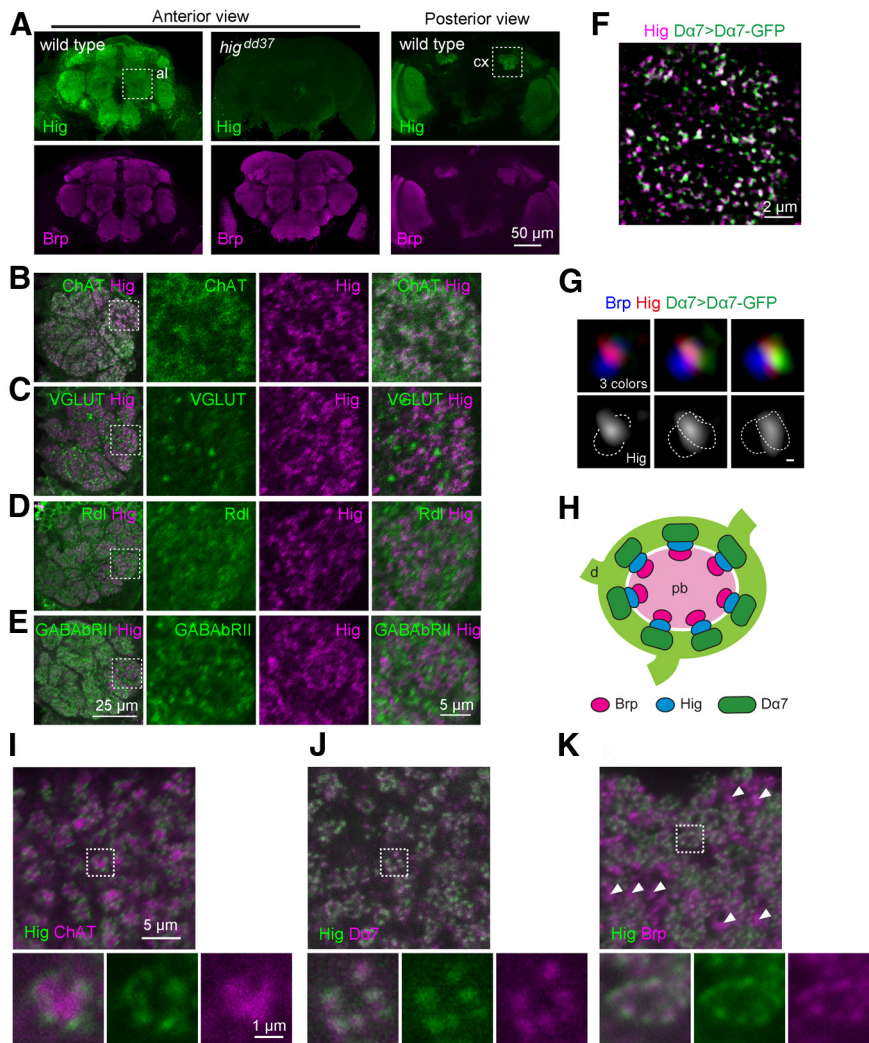
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**Figure 1.** Hig localizes in the synaptic clefts of cholinergic synapses in the brain. **A**, Distribution of Hig in the brain. Frontal sections of the anterior and posterior brain regions are shown. Hig was observed in the entire synaptic region labeled with Brp in the WT brain, whereas Hig signals disappeared in *hig<sup>dd37</sup>*. al, Antennal lobe; cx, calyx of the MB. **B–E**, Double labeling of synapses in the antennal lobe with antibodies against synaptic proteins and Hig. Hig was closely associated with ChAT (**B**) but not VGLUT (**C**), Rdl (**D**), or GABA<sub>B</sub>RII (**E**). Single glomeruli surrounded by dotted squares are magnified in the three right panels. **F, G**, High-resolution images of synapses in the antennal-lobe glomerulus obtained by structured illumination microscopy. Each Hig-positive synapse in focus is colabeled with Dα7-GFP, driven by *Dα7-GAL4* (**F**). In most synapses, Hig (red at the top, gray at the bottom) localizes between Brp (blue) and Dα7-GFP (green) (**G**). Serial optical images were obtained at Z intervals of 0.3 μm. Scale bar, 100 nm. **H**, Schematic model for the distribution of Brp, Hig, and Dα7 in a microglomerulus formed by a single presynaptic bouton (pb) of olfactory projection neurons and the dendrites (d) of MB Kenyon cells (for the structure of the microglomerulus and distribution of synaptic markers, see Kremer et al., 2010). **I–K**, Distribution pattern of Hig in microglomeruli of the calyx. The single microglomerulus in a dotted square is enlarged in the three lower panels. Most Hig-positive microglomeruli were associated with ChAT (**I**). Discrete signals of Hig in microglomeruli were juxtaposed with Dα7 (**J**) and the active-zone marker Brp (**K**). **K**, Arrowheads indicate synapses without Hig, which are not cholinergic.

pCasperHS-higD30GFP, which contains the longest form of *hig* cDNA fused with GFP. The resulting fragment was cloned into the EcoRI site of pUASTattB (Bischof et al., 2007). Transgenic fly lines expressing *UAS-hig-GFP* were generated by Phi-C31-mediated site-specific integration at attP2, P18, and P40.

**Antibody production.** To generate anti-Hig antibody, a fragment of *hig* cDNA encoding the N-terminal fragment (amino acids 40–266) was amplified by PCR from GH23986 (BDGP) and cloned into pET15b (Novagen). Hexahistidine fusion proteins were expressed in *Escherichia coli* BL21 (DE3), purified, and injected into guinea pigs. To generate anti-Dα6 antibody, DNA encoding the cytoplasmic loop (amino acids 392–460) was amplified by PCR from GH15518 (BDGP) and cloned into pGEX4T-1 (GE Healthcare). GST fusion proteins were expressed in *E.*

*coli* BL21 (DE3), purified, and injected into rabbits. Anti-Hig and anti-Dα6 antibodies were purified by affinity binding to the respective antigens.

**Immunohistochemistry.** Brains of adult male flies were dissected in PBS, fixed with 4% paraformaldehyde (PFA) in PBS on ice for 1 h, and stained with the following antibodies: anti-Hig (1:1000), anti-Bruchpilot (nc82, 1:20, DSHB), anti-ChAT (ChAT4B1, 1:20, DSHB), anti-DVGLUT (1:500, Mahr and Aberle, 2006), anti-Rdl (1:1000, Enell et al., 2007), anti-GABA<sub>B</sub>RII (1:1000, Enell et al., 2007), anti-DLG (4F3, 1:100, DSHB), anti-DN-cadherin (Ex#7, 1:20, DSHB), or anti-GFP (1:500, Invitrogen). AlexaFluor (-488, -568, -594, and -647)-conjugated antibodies (1:200, Invitrogen) were used as secondaries. For staining with anti-Dα7 (1:1000) and anti-Dα6 (1:1000), dissected brains were fixed with 4% PFA in PBS for 10 min on ice. Samples were observed by sequential scans on an SP2 confocal microscope (Leica) or ELYRA S.1 microscope (Zeiss). Quantitation of confocal images was performed using the Leica confocal software, with fluorescence intensity compared with that of control samples stained simultaneously in the same tube. At least seven samples were examined for the purposes of statistical analysis.

**Insecticide assay.** Insecticide resistance assays were performed as previously reported (Watson et al., 2010) with slight modifications. Spinosad was dissolved at appropriate concentrations in 2:1 acetone:10% sucrose solution, and then 0.1 ml of each solution was applied to vials (inner diameter 2.1 cm) containing 5 ml of blue agar (1% agar, 1% Brilliant Blue FCF). Vials were dried overnight. Ten male flies (3- to 5-d-old) were introduced to each vial, and mortality was examined over 24 h. To assess the amount of food ingested by surviving flies, the bodies were separated from heads, homogenized in PBS, and centrifuged at 13,000 rpm for 25 min. After the supernatants were recentrifuged, absorbance was measured at 625 nm.

## Results

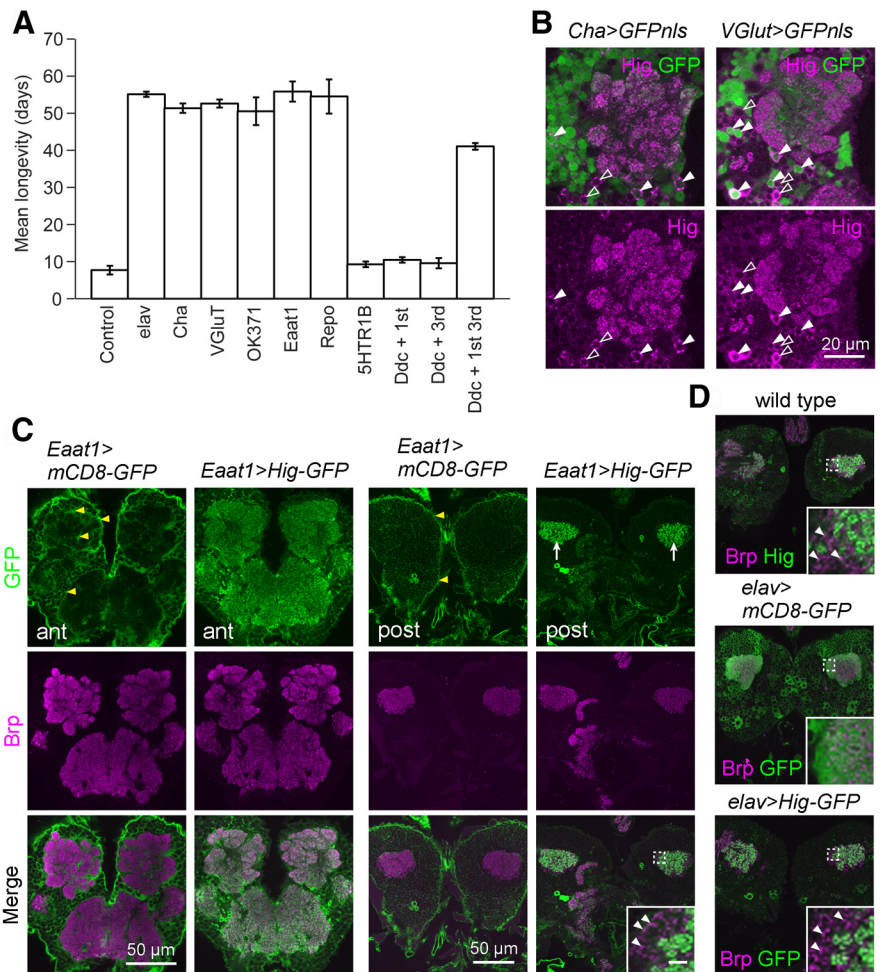
### Hig localizes predominantly at cholinergic synapses

Hig was detected most abundantly in synaptic regions that labeled positively for the presynaptic active-zone protein Bruchpilot (Brp; Wagh et al., 2006) in the adult brain (Fig. 1A). This Hig signal disappeared in the *hig<sup>dd37</sup>* mutant *hig<sup>dd37</sup>*, confirming the specificity of the anti-Hig antibody (Fig. 1A). To identify the neurotransmitter associated with the Hig-positive synapses, we performed double immunolabeling for several synaptic markers and Hig (Fig. 1B–E). The antennal lobes are well-analyzed regions containing synapses that use acetylcholine, glutamate, and GABA as neurotransmitters (Liu and Wilson, 2013). Synaptic Hig was closely associated with ChAT, a presynaptic marker of cholinergic neurons, whereas vesicular glutamate transporter (VGLUT) and GABA receptors (Rdl and GABA<sub>B</sub>RII) were largely excluded from Hig-positive zones (Fig. 1B–E). To confirm the distribution of Hig at cholinergic synapses, we used structured illumination microscopy to obtain

high-resolution images of synapses, taking advantage of the surface localization of the antennal lobes in the brain. We expressed *Da7-GFP*, a GFP-tagged AchR subunit *Da7*, using a *Da7-GAL4* driver line; when the center of each signal was focused, the *Da7-GFP* signals were closely associated with Hig (Fig. 1F). Structured illumination microscopy also revealed fine images of a single synapse in which Hig was present between the presynaptic marker Brp and the postsynaptic marker *Da7-GFP*, indicating that Hig was localized to the synaptic cleft (Fig. 1G). The microglomerulus of the mushroom body (MB) calyx is a specialized synaptic complex that uses acetylcholine as a neurotransmitter (Fig. 1I, J) (Yasuyama et al., 2002) and is surrounded by noncholinergic synapses (Fig. 1K). This complex comprises an axon terminal of the projection neuron at its center and the dendrites of MB Kenyon cells (Fig. 1H) (Kremer et al., 2010). In most microglomeruli, a discrete pattern of Hig staining was observed in the surrounding regions of ChAT-positive projection neuron axon terminals (Fig. 1I). These immunohistochemical data from antennal lobe, MB, and other brain regions, including the optic lobes (see Fig. 3A, B), indicated that Hig predominantly localized to cholinergic synapses in the brain. To further analyze the discrete pattern of Hig in the microglomeruli, brains were simultaneously stained for *Da7* and Hig or Brp and Hig. Hig did not simply diffuse over the entire space of the synaptic clefts but was instead juxtaposed with *Da7* on the postsynaptic membrane (Fig. 1H, J); it also colocalized with Brp, an active-zone marker in the presynaptic terminal (Fig. 1H, K). We conclude that Hig is predominantly distributed in the cholinergic synapses and localizes at the synaptic cleft region adjacent to the active zone.

### Hig is extracellularly diffused and trapped by cholinergic synapses

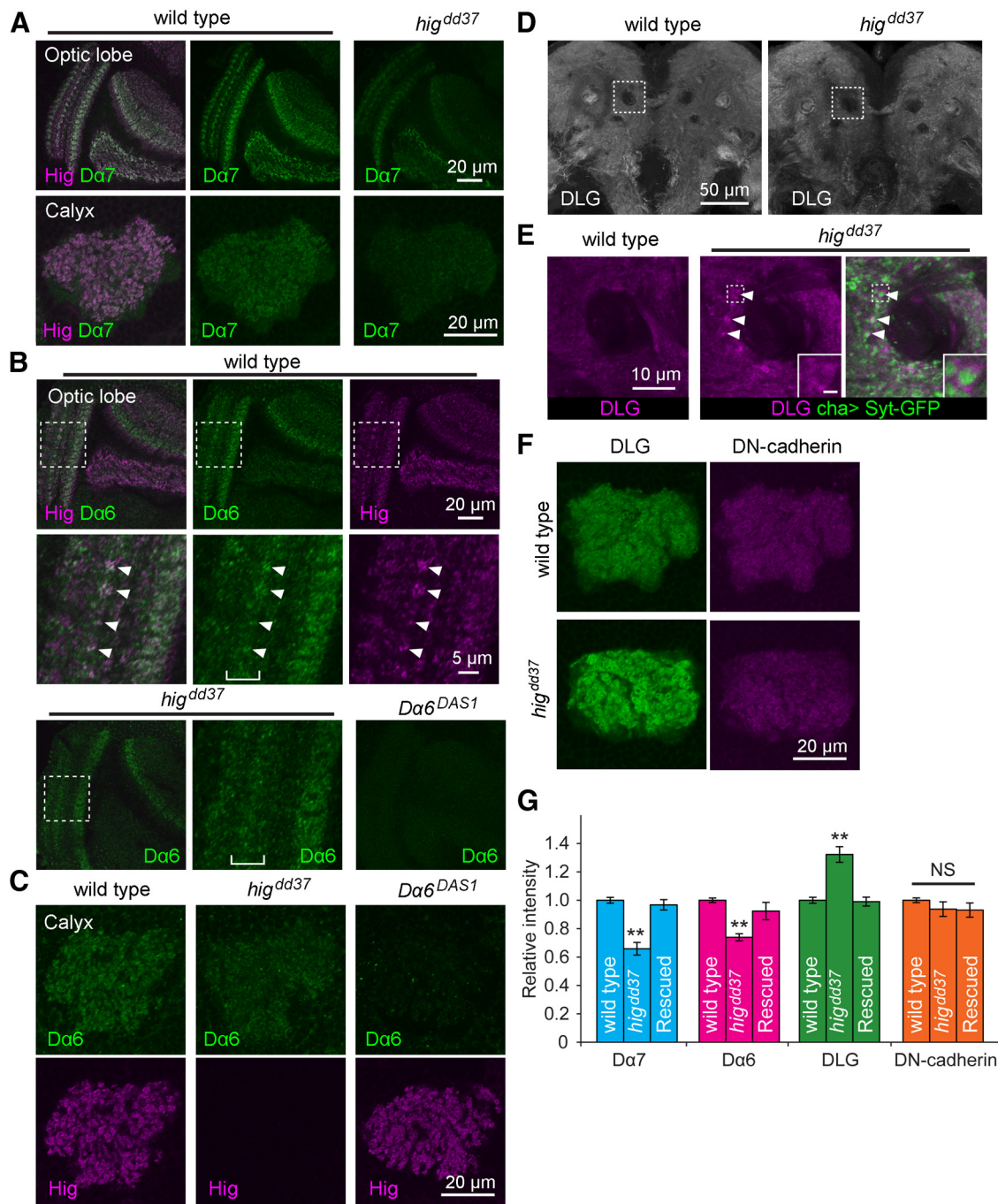
Because Hig is predominantly distributed in the cholinergic synapses, we predicted that the *hig* mutant phenotype could be rescued by expression of transgenic *hig* in the cholinergic neurons. To test this idea, we expressed GFP-tagged Hig (Hig-GFP) in the *hig<sup>dd37</sup>* background under the control of several GAL4 drivers. The *hig<sup>dd37</sup>* homozygotes exhibited reduced longevity, and this phenotype was rescued by expression of Hig-GFP in all neurons (*elav-Gal4*) and cholinergic neurons (*Cha-Gal4*), as expected (Fig. 2A). However, the longevity of *hig<sup>dd37</sup>* was also rescued by *hig* expression in glutamatergic neurons (*OK371-Gal4* and *VGlut-Gal4*) as well as in glial cells (*Eaat-Gal4* and *Repo-Gal4*) (Fig. 2A). By contrast, Gal4 lines driving expression in a small number of neurons (*5HTR1B-Gal4*, Yuan et al., 2005; *Ddc-Gal4*, Liu et al., 2012) were unable to rescue the mutant phenotype.



**Figure 2.** Secreted Hig-GFP is captured by the cholinergic synapses. **A**, Rescue of the *hig* phenotype by ectopic expression of Hig-GFP. The indicated GAL4 lines were used to drive Hig-GFP expression in cholinergic, glutamatergic, or dopaminergic neurons, or glia, in the *hig* mutant background. The genotype of the control is *hig<sup>dd37</sup>; UAS-hig-GFP/+; UAS-hig-GFP* on the third chromosome was used for most of the experiments, unless otherwise stated. *Ddc + 1st: UAS-hig-GFP/Y; Ddc-Gal4, hig<sup>dd37</sup>/hig<sup>dd37</sup>; +/+*. *Ddc + 3rd: +/Y; Ddc-Gal4, hig<sup>dd37</sup>/hig<sup>dd37</sup>; UAS-hig-GFP/+*. *Ddc + 1st 3rd: UAS-hig-GFP/Y; Ddc-Gal4, hig<sup>dd37</sup>/hig<sup>dd37</sup>; UAS-hig-GFP/+*. Error bars indicate  $\pm$  SEM. **B**, Expression of endogenous Hig in cholinergic and glutamatergic neurons. Cholinergic and glutamatergic neurons were labeled with nuclear GFP driven by *Cha-Gal4* and *VGlut-Gal4*, respectively. Filled and open arrowheads indicate Hig-expressing neurons with and without GFP signals, respectively. **C**, Synaptic localization of Hig-GFP ectopically expressed in the glia. *Eaat1-GAL4* was used for glial expression of membrane-bound mCD8-GFP and Hig-GFP. Hig-GFP is detected in most synaptic regions labeled with Brp in both the anterior (ant) and posterior (post) brain sections, whereas mCD8-GFP labels glial membranes (yellow arrowheads). In MB calyx of the posterior section, Hig-GFP was detected in cholinergic microglomeruli (arrows) but excluded from noncholinergic synapses outside microglomeruli (inset, arrowheads). Scale bar: **C**, inset, 5  $\mu$ m. **D**, Microglomerulus-specific localization of endogenous Hig and pan-neuronally expressed Hig-GFP. As in **C**, inset, endogenous Hig (top) and Hig-GFP driven by *elav-Gal4* (bottom) were only detected in cholinergic microglomeruli, but not in other synapses (arrowheads). mCD8-GFP driven by *elav-Gal4* labeled the whole synaptic region of the calyx (middle).

Nevertheless, when expression of Hig-GFP was enhanced by addition of one more copy of the *hig* transgene, *Ddc-Gal4* could prolong longevity (Fig. 2A). These results indicate that Hig does not need to be expressed in cholinergic neurons. Indeed, endogenous Hig was detected in the cell bodies of a large number of glutamatergic and cholinergic neurons (Fig. 2B).

The results of these rescue experiments and the synaptic distribution of Hig suggested that secreted and diffused Hig is eventually integrated into cholinergic synaptic clefts. To test this possibility, we examined the distribution of Hig-GFP expressed by a glia-specific driver (Fig. 2C). Secreted Hig-GFP was detected in most synaptic regions throughout the brain, notably in the cholinergic microglomeruli of the MB calyx (Fig. 2C), whereas Hig-GFP was not detected in noncholinergic synapses outside of



**Figure 3.**  $D\alpha 7$  and  $D\alpha 6$  levels decrease, but DLG level increases in the synaptic regions of adult *hig<sup>dd37</sup>* brain. **A**, Reduction of  $D\alpha 7$  localization at synapses of the optic lobe (top) and calyx microglomeruli (bottom) in *hig<sup>dd37</sup>*. Hig was well colocalized with  $D\alpha 7$ . **B**, Decrease of  $D\alpha 6$  localization at subsets of synapses in the optic lobe of *hig<sup>dd37</sup>*. Magnified views of dotted areas of the medulla are shown in the middle panels for WT, and in the bottom center panel for *hig<sup>dd37</sup>*. Strong Hig staining is found in specific layers of synapses that also contain  $D\alpha 6$  signals (arrowheads).  $D\alpha 6$  in these synapses (bracket) is significantly decreased in *hig<sup>dd37</sup>*.  $D\alpha 6$  signals disappeared in *D\alpha 6<sup>DAS1</sup>* mutant brains, confirming the specificity of the anti- $D\alpha 6$  antibody. **C**, Reduction in  $D\alpha 6$  signals at the synapses of the calyx microglomeruli in *hig<sup>dd37</sup>*. **D–F**, Abnormal accumulation of DLG in *hig<sup>dd37</sup>*. **D**, Frontal optical sections of WT and *hig<sup>dd37</sup>* brains stained for DLG, which labels synapses. The dotted square in the synaptic region is enlarged in **E**. **E**, Abnormal accumulation of DLG at cholinergic synapses in *hig<sup>dd37</sup>*. Strong DLG signals (arrowheads) are adjacent to the presynaptic marker Syt-GFP, expressed under the control of *cha-Gal4* (dotted squares, and insets for magnified views). Scale bar: **E**, inset, 1  $\mu$ m. **F**, Abnormal accumulation of DLG in the calyx of *hig<sup>dd37</sup>*. The intensity of DN-cadherin in *hig<sup>dd37</sup>* is comparable with that in the WT. **G**, Quantitative analyses of synaptic proteins  $D\alpha 7$ ,  $D\alpha 6$ , DLG, and DN-cadherin in the calyx of WT, *hig<sup>dd37</sup>*, and rescued flies (*elav-Gal4/Y; hig<sup>dd37</sup>; UAS-hig-GFP/+*). Intensity of fluorescence in *hig<sup>dd37</sup>* is normalized to the WT values. Error bars indicate  $\pm$  SEM. \*\* $p < 0.01$ , compared with WT (ANOVA followed by Bonferroni's *post hoc* test). NS, Not significantly different.

the microglomeruli (Fig. 2C, inset). This microglomerulus-specific localization was also observed for endogenous Hig and Hig-GFP driven by *elav-Gal4* in all neurons (Fig. 2D, insets). Thus, Hig is produced in cholinergic and noncholinergic neurons, secreted to extracellular spaces, and finally trapped in cholinergic synaptic clefts by a specific mechanism.

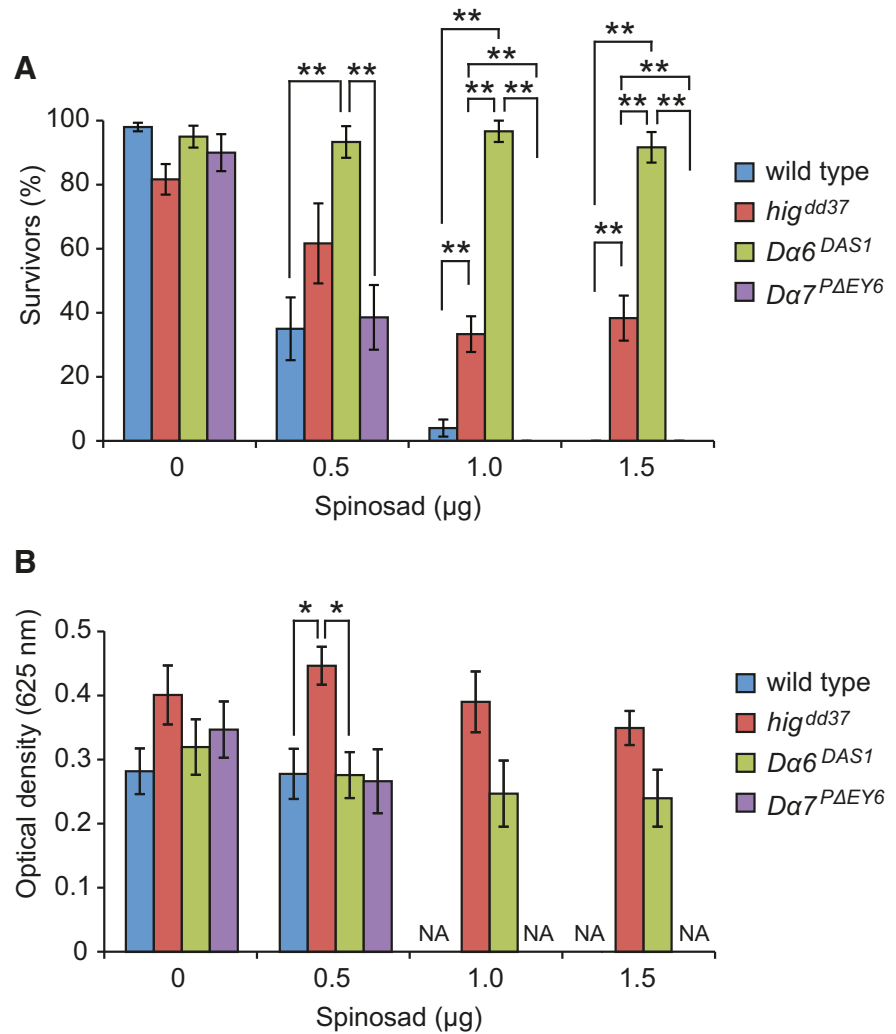
### Hig regulates the synaptic distribution of $D\alpha 7$ , $D\alpha 6$ , and DLG

Because Hig is required for normal locomotor activity and longevity, we predicted that the neural function of cholinergic synapses would be partly impaired in the *hig* mutant. We therefore examined the distribution of several proteins localized to cholinergic synapses.

ergic synapses in the *hig<sup>dd37</sup>* brain. Although no obvious changes in the distribution of ChAT and acetylcholine esterase were observed in *hig<sup>dd37</sup>* brains (data not shown), levels of the AchR subunit  $\text{D}\alpha 7$ , which was well colocalized with Hig in most brain regions, were significantly reduced throughout the brain, including MB calyx (Fig. 3A,G). In addition, levels of another AchR subunit  $\text{D}\alpha 6$  were reduced;  $\text{D}\alpha 6$  colocalized with Hig in many regions, including the layers of the medulla neuropil of the optic lobe and the MB calyx (Fig. 3B,C,G). Clustering of neurotransmitter receptors on the postsynaptic membranes is mediated by cytoskeletal scaffold proteins (Renner et al., 2008). Therefore, we examined the distribution of DLG, a *Drosophila* ortholog of PSD-95. At low magnification, the overall staining pattern of DLG in most brain regions did not differ significantly between wild-type (WT) and *hig* mutants (Fig. 3D). However, a higher-magnification view revealed elevated fluorescent intensity of DLG staining in a punctate pattern throughout the brain; each of the puncta was juxtaposed with the presynaptic terminals of cholinergic neurons (Fig. 3E). More conspicuously, DLG accumulated abnormally at microglomeruli in the MB calyx of *hig<sup>dd37</sup>*, although the level of DN-cadherin was unchanged (Fig. 3F,G). Accumulation of  $\text{D}\alpha 7$ ,  $\text{D}\alpha 6$ , and DLG in *hig<sup>dd37</sup>* brains was restored to WT levels by pan-neuronal expression of Hig-GFP (Fig. 3G). These results indicate that Hig is required for the proper accumulation of AchR subunits and the scaffold protein DLG in postsynaptic terminals of cholinergic synapses.

#### *hig* mutant flies are resistant to a $\text{D}\alpha 6$ agonist

Because synaptic accumulation of  $\text{D}\alpha 7$  and  $\text{D}\alpha 6$  was reduced in the *hig<sup>dd37</sup>* mutant, we predicted that phenotypes similar to those of  $\text{D}\alpha 7$  and  $\text{D}\alpha 6$  mutants may occur in *hig<sup>dd37</sup>*. Spinosad is a  $\text{D}\alpha 6$  agonist that specifically binds  $\text{D}\alpha 6$  and causes lethality by continuously activating AchR channels. Therefore, a null mutant of the  $\text{D}\alpha 6$  gene,  $\text{D}\alpha 6^{\text{DAS1}}$ , is highly resistant to spinosad (Fig. 4A) (Watson et al., 2010). When flies were reared on medium containing 1.0 or 1.5  $\mu\text{g}$  of spinosad on the surface, *hig<sup>dd37</sup>* mutants survived at significantly higher frequencies than the control strains (WT and  $\text{D}\alpha 7^{\text{P}\Delta\text{EY6}}$ ), but at lower frequencies than  $\text{D}\alpha 6^{\text{DAS1}}$  flies (Fig. 4A). One potentially confounding factor was that, because *hig<sup>dd37</sup>* flies have reduced locomotor activity, they may eat smaller amounts of food than more active strains. To test this, we investigated the intake of food by measuring the amount of dye in extracts from surviving flies. Somewhat surprisingly, *hig<sup>dd37</sup>* flies tended to take even a larger amount of food than WT and  $\text{D}\alpha 6^{\text{DAS1}}$ , regardless of the content of spinosad (Fig. 4B); thus, the feeding behavior of *hig<sup>dd37</sup>* flies was not reduced. These data collectively indicate that *hig<sup>dd37</sup>* flies are partly resistant to spinosad, consistent with the notion that *hig<sup>dd37</sup>* impairs the cho-



**Figure 4.** *hig<sup>dd37</sup>* flies exhibit partial resistance to spinosad. **A**, Test for spinosad resistance. Flies (3- to 5-d-old) were reared on media with the indicated amount of spinosad for 24 h, and surviving flies were counted for each genotype. **B**, Feeding activity was estimated by measuring the optical absorbance of the dye in extracts from surviving flies. NA, Not applicable. Error bars indicate  $\pm$  SEM. The resistance test was performed at least five times (50 flies) for each genotype. \* $p < 0.05$  (ANOVA followed by Bonferroni's *post hoc* test). \*\* $p < 0.01$  (ANOVA followed by Bonferroni's *post hoc* test).

linergic synaptic function mediated by  $\text{D}\alpha 6$ . Thus, the matrix protein Hig organizes a molecular architecture involving  $\text{D}\alpha 6$ ,  $\text{D}\alpha 7$ , and DLG in the postsynaptic terminals, and mutants defective in this organization exhibit reduced locomotion and longevity due impairments in the function of cholinergic synapses.

#### Discussion

The data of Hig presented here provide the first evidence in any organism of a synaptic matrix protein specific to cholinergic synapses of the CNS. Hig secreted from cholinergic and noncholinergic neurons diffuses but is specifically trapped at the cholinergic synaptic clefts and then organizes a molecular architecture, including DLG and AchR subunits  $\text{D}\alpha 6$  and  $\text{D}\alpha 7$  in postsynaptic terminals.

In *hig* mutant brains, the intensity of antibody staining for  $\text{D}\alpha 6$  and  $\text{D}\alpha 7$  was notably reduced in the synaptic regions. Consistent with this result, *hig* mutant flies were resistant to a  $\text{D}\alpha 6$  agonist, spinosad. Therefore,  $\text{D}\alpha 6$ -mediated neurotransmission is most likely to be impaired in *hig* flies, and deficits in  $\text{D}\alpha 6$  as well as  $\text{D}\alpha 7$  might explain why the mutant flies exhibited reduced locomotion and longevity. However, double-null mutants of

$D\alpha 6$  and  $D\alpha 7$  exhibit apparently normal locomotor behavior (data not shown), suggesting that *hig* mutant phenotypes are caused by additional defects in other factors, including AchR subunits, that functionally overlap with  $D\alpha 6$  and  $D\alpha 7$ . Among 10 nAChR subunits encoded by the *Drosophila* genome,  $D\alpha 5$  can be combined with  $D\alpha 6$  and  $D\alpha 7$  to form functional heteromeric channels and also forms a functional homomeric channel (Lansdell et al., 2012). Therefore,  $D\alpha 5$  may compensate for loss of  $D\alpha 6$  and  $D\alpha 7$  in double mutants and is therefore a candidate for the AchR subunit responsible for reduced locomotion and longevity of *hig* flies.

Hig contains CCP domains, which are found in proteins that regulate complement activity in the vertebrate immune system. However, many CCP proteins exist in the nervous system of vertebrates and invertebrates, suggesting that they play evolutionarily conserved roles in neural function or development. Some of these proteins have been reported to localize at specific synapses and function in their differentiation (Nakayama and Hama, 2011). In *C. elegans*, the CCP proteins LEV-9 and AchR depend on each other for proper synaptic localization, and the two proteins are required for normal development of the NMJ (Gendrel et al., 2009). The human CCP protein SRPX2 is associated with rolandic epilepsy and oral dyspraxia (Roll et al., 2006), and its mouse counterpart regulates formation of glutamatergic synapses in the brain (Sia et al., 2013). When these CCP proteins modulate the development of synapses, a series of CCP domains in a polypeptide possibly enables it to interact with other proteins and participates in construction of the scaffolds of the synaptic clefts. CCP proteins have been shown to form a complex with proteins containing CCP or CUB domains (Tuveson et al., 1991; Gendrel et al., 2009). Therefore, some proteins with these domains in *Drosophila* may be Hig binding partners that are responsible for incorporation of Hig into specific synaptic clefts. Further studies of Hig should reveal the molecular composition of the synaptic cleft matrix, which gathers extracellular and membrane proteins on the terminal structures during cholinergic synaptogenesis. Because many CCP proteins are present in the human brain, the results of such investigations may be of clinical relevance.

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