Mutation of *Drosophila homer* Disrupts Control of Locomotor Activity and Behavioral Plasticity

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Homer proteins have been proposed to play a role in synaptogenesis, synapse function, receptor trafficking, and axon pathfinding. Here we report the isolation and characterization of the *Drosophila* gene *homer*, the single Homer-related gene in fly. Using anti-Homer antibody we show that Homer is expressed in a broad range of tissues but is highly enriched in the CNS. Similarly to its mammalian counterpart, the *Drosophila* Homer localizes to the dendrites and the endoplasmic reticulum (ER). This subcellular distribution is dependent on an intact Enabled/Vasp homology 1 domain, suggesting that Homer must bind to one or more of its partners for proper localization. We have

created a mutation of *homer* and show that flies homozygous for this mutation are viable and show coordinated locomotion, suggesting that Homer is not essential for basic neurotransmission. However, we found that *homer* mutants display defects in behavioral plasticity and the control of locomotor activity. Our results argue that in the CNS, Homer-related proteins operate in the ER and in dendrites to regulate the development and function of neural networks underlying locomotor control and behavioral plasticity.

Key words: *Drosophila*; *Homer*; dendrites; locomotion; courtship; behavioral plasticity

Proteins of the Homer family have been implicated in synaptogenesis, signal transduction, receptor trafficking, and axon pathfinding (for review, see Xiao et al., 2000; Foa et al., 2001). In mammals, there are three independent genes, Homer-1, -2, and -3, which through differential splicing encode at least six Homer proteins (Kato et al., 1998; Xiao et al., 1998). Members of the Homer family are expressed in various tissues but appear to be enriched in the CNS, where they have partially overlapping domains of expression (Brakeman et al., 1997; Xiao et al., 1998). Homer proteins are bipartite, consisting of an N-terminal Enabled/Vasp homology 1 (EVH1) domain and a C-terminal coiled-coil (CC) domain that mediates self-association (Brakeman et al., 1997; Kato et al., 1998; Tu et al., 1998; Xiao et al., 1998; Tadokoro et al., 1999). The EVH1 domain binds to group I metabotropic glutamate receptors (mGluRs) and their downstream effectors, the inositol-triphosphate receptor (InsP3R), by interacting with a proline-rich motif (PPxxF) found in these proteins (Brakeman et al., 1997; Tu et al., 1998, 1999).

One can envision a model in which Homer proteins, via their

ability to self-associate, modulate group I mGluR function by mediating the formation of a multimolecular complex required for local and fast increase of Ca^{2+} concentration during mGluR activation (Xiao et al., 2000). Supporting this notion is the finding that Homer proteins regulate the intracellular trafficking of mGluRs (Roche et al., 1999; Ango et al., 2000). Further modulation is provided by Homer 1a, one of the proteins encoded by the Homer 1 gene that consists of the EVH1 domain without the CC domain required for multimerization. Homer 1a is upregulated during synaptic activity (Brakeman et al., 1997; Xiao et al., 1998) and is capable of attenuating mGluR-evoked intracellular calcium release *in vitro*, presumably by disruption of a putative mGluR–Homer–InsP3R multimeric complex (Tu et al., 1998).

Homer proteins also bind to Shank/ProSAP, a postsynaptic protein that is part of a complex including the NMDA-type glutamate receptors (Boeckers et al., 1999; Naisbitt et al., 1999; Tu et al., 1999). Recently, Shank has been implicated in the regulation of dendritic spine morphology and synaptic function, and this regulation is dependent on Shank binding to Homer (Sala et al., 2001). Therefore, Homer-related proteins may be part of a large multimolecular complex that modulates the structural and functional plasticity of glutamatergic synapses (Naisbitt et al., 1999; Sala et al., 2001).

In the absence of mutations in any of the mammalian Homer genes, their *in vivo* roles remain unknown. We and others (Kato et al., 1998; Xiao et al., 1998) have identified a single gene in *Drosophila* encoding a protein homologous to the mammalian Homer proteins. Here, we show that *Drosophila* Homer is enriched in the nervous system where it is localized to the endoplasmic reticulum (ER) and targeted to dendritic processes. In addition, we provide genetic evidence that *homer* is required for the function of the neural networks controlling locomotor activity and behavioral plasticity.

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MATERIALS AND METHODS

Fly strains and genetics. The EP strain EP(2)2141 (Rorth, 1996) was obtained from the Berkeley *Drosophila* Genome Project collection. The *homer*⁺ precise excision and *homer*^{R102} imprecise excision were generated by mobilization of the EP(2)2141 P-element using $\Delta 2,3$ as a source of transposase (Tsubota and Schedl, 1986; Robertson et al., 1988). We identified another transcription unit, 700 bp upstream of the first exon of *homer* and in the opposite orientation, that encodes a predicted protein with homology to the Liprin gene family (Serra-Pages et al., 1998). Using an antibody against DLiprin (kindly provided by N. Kaufmann and D. van Vactor, Harvard Medical School, Boston, MA), we verified that the expression of the neighboring *DLiprin* gene was not affected in *homer*^{R102} mutant flies. The *homer*⁺ and *homer*^{R102} chromosomes were balanced, and the first and third chromosomes were then exchanged for Canton-S wild-type chromosomes. All crosses and embryo collections were performed at 25°C.

DNA constructs. We obtained the LD03156 cDNA that encodes full-length Homer (Research Genetics Inc.). To create Homer-myc, we used PCR to fuse six copies of the c-myc epitope to the C terminus of Homer cDNA. The mutEVH-Homer mutations were introduced into Homer-myc by PCR using the following primers: mutEVH-1, CGGGGAAT-CACGGTGTACCACTTGCATGCTGCTTCAGAG; mutEVH-2, CTCTGAAGCAGCATGCAAGTGGTACACCGTGATCCCCG.

Homer Δ C contains only the first 175 aa and was created by making use of the intrinsic *HindIII* site after which one myc epitope and a stop codon were inserted. Homer-myc, mutEVH-Homer-myc, and Homer Δ C were each verified by sequencing and then cloned into pUAST (Brand and Perrimon, 1993). The UAS lines were generated by germline transformation (Rubin and Spradling, 1982). For each construct, several lines were created and tested for expression.

Yeast two-hybrid assay. The Homer bait was constructed by cloning a PCR fragment encoding amino acids 1–186 of Homer in frame with the GAL4 DNA binding domain using the pGBT9 vector (Clontech). The mutEVH bait was constructed similarly except that the EVH domain was mutated using the same primers used for the construction of mutEVH-Homer-myc. *Drosophila* Shank was identified in a screen of a *Drosophila* λ ACT2 embryonic library (gift from S. Elledge, Baylor College of Medicine, Houston, TX) with the EVH1 domain of Homer as a bait. This Shank cDNA encodes the last 114 amino acids of the *Drosophila* Shank protein (GenBank accession number AY047554). The *Drosophila* mGluRA prey was generated by cloning a PCR fragment encoding the last 130 amino acids of DmGluRA in frame with the GAL4 activation domain in the pGAD424 vector (Clontech).

We transformed the yeast strain AH109 (Clontech) following the protocol described by the manufacturer and selected double transformants on media lacking leucine and tryptophan. We tested the bait/prey interaction on triple selection media lacking either adenine or histidine.

Homer antibody production and Western blot analysis. We generated a bacterially expressed glutathione S-transferase (GST)-fusion protein by cloning a PCR fragment encoding amino acids 2–204 of Homer in the vector pGEX4T-2 (Amersham Biosciences). The affinity-purified fusion protein was used to immunize rats. Homer and Discs-large (DLG) immunodetection on Western blot were performed as described previously (Thomas et al., 2000). Rat anti-Homer serum was used at a 1:4000 dilution. Horseradish peroxidase (HRP)-conjugated secondary antibodies were used at a 1:10,000 dilution.

In situ hybridization and immunostaining. *In situ* hybridization was performed as described previously (Bourgouin et al., 1992). We used a digoxigenin (DIG)-labeled full-length antisense cDNA probe synthesized from the LD03156 Homer cDNA. A control sense probe did not yield a signal above background level. Embryo dissections and HRP immunostaining were performed as described previously (Callahan and Thomas, 1994). For immunofluorescence staining, we used secondary antibodies conjugated to Cy3 or Cy5 (Jackson ImmunoResearch) or FITC (Vector Laboratories) at a dilution of 1:500. The primary antibodies were used at the following dilutions: mouse anti-myc (9E10) 1:50; mouse anti-Golgi 1:75 (Calbiochem 345865); mouse anti-BiP 1:100 (StressGen SPA-827); FITC-conjugated goat anti-HRP 1:400; mouse mAb 22C10 1:50; anti-Fasciclin II 1:50; mAb BP102 1:50; rabbit anti-Synaptotagmin 1:1000. Rat anti-Homer was first preabsorbed overnight at 4°C with *homer* mutant embryos and then used at a concentration of 1:1000. Confocal analysis was performed on a Zeiss confocal station and imaged with the LSM510 software (Zeiss). Images were compiled with the Adobe Photoshop 5.0.

Courtship assays. All males were 5–7 d old on the day of testing. Each

male was kept isolated shortly after hatching and therefore had no sexual encounters before the courtship assays. The males were coded and the observer (T.T.D.) was blind to the genotype when monitoring courtship activity. Females (mated or virgin) were 4–5 d old and were of *w*¹¹¹⁸ genotype. The mated females were mated 1 d before the assay and kept separated from males until testing. Trained males were placed with a mated female for 1 hr in an observation chamber (8 mm in diameter, 3.5 mm in depth). The mated female was then carefully removed, and the male was left alone for 2 min before an anesthetized virgin female was gently introduced into the chamber. Over a total period of 10 min, the time in which the male was engaged in courtship was measured when the male displayed the following behaviors: orientation toward the female, tapping or licking the female, wing extension, and attempts to copulate. For the naive males, the testing conditions were the same except that they were left alone in the chamber for 62 min before the introduction of the virgin female. For the monitoring of the decrement of courtship, the conditions were the same as for the trained males described above; courtship was monitored over the first and last 10 min of 1 hr with the mated female. The experiment was aborted if the male did not show at least 1 min of courtship in the first 10 min of the experiment.

All of the statistical analysis of the courtship data was performed using Statview (SAS Inc.). We used the Kruskal–Wallis nonparametric rank sums tests for group comparisons and the Dunn test for multiple comparisons (Zar, 1984). For pair-wise comparisons of courtship measurements, we used the nonparametric Mann–Whitney rank sum test and the sign test for correlated data.

Olfactory and locomotor tests. The chemosensory jump assay was performed as described elsewhere (McKenna et al., 1989). Locomotor tests were performed as described previously (Kane et al., 1997) using the same chambers as those used for the courtship assay. For the statistical analysis of spontaneous locomotion data, we used the Mann–Whitney rank sum test (Statview).

RESULTS

Characterization of the *Drosophila homer* gene

In the Berkeley *Drosophila* Genome Project sequence database, we identified several expressed sequence tags (ESTs) encoding a gene product with high homology to the mouse protein Homer 1. DNA sequencing revealed that these different cDNAs encode a predicted protein of 394 amino acids. In this report we used the *Drosophila* FlyBase nomenclature and referred to this gene as *homer*. The same ESTs have been identified previously, and the predicted gene product was referred to as either D-Homer or Dvh (*Drosophila* Ves-1 homolog) (Kato et al., 1998; Xiao et al., 1998).

The N-terminal 120 amino acids of *Drosophila* Homer, which contain the EVH1 domain, show 73% amino acid identity to the rodent Homer 1 proteins. Although the *Drosophila* Homer C-terminal region overall shows only 25% identity with the Homer 1b protein, there are conserved amino acids within the CC domain and the two putative leucine zippers, which are thought to be involved in multimerization of Homer proteins (Tu et al., 1998; Xiao et al., 1998; Tadokoro et al., 1999). Alignment of *homer* cDNA and genomic sequences predicts a gene structure of seven exons (Fig. 1A).

Because Homer 1 is a member of a family of at least three genes in mouse (Xiao et al., 1998), we wondered whether there might be other Homer-related genes in *Drosophila*. Homology searches of the *Drosophila* genomic sequence database failed to reveal any additional Homer-related genes. In addition, we were unable to identify any other Homer-related genes by low stringency genomic Southern blots with a *homer* cDNA probe or by degenerate RT-PCR. Therefore, it appears that there is a single Homer-related gene in *Drosophila*.

homer RNA is highly enriched in the embryonic nervous system

To investigate the spatial and temporal regulation of *homer* expression, we performed whole-mount *in situ* hybridization of a

Figure 1. Genomic structure and expression of *homer*. *A*, Genomic organization of the *homer* locus. Black and open boxes indicate the translated and untranslated sequences, respectively, of Homer cDNA. Restriction sites for *Bam*HI (*B*), *Hind*III (*H*), *Pac*I (*P*), and *Xho*I (*X*) are indicated. The breakpoints for the *homer*^{R102} deletion caused by imprecise excision of the [EP(2)2141] P-element are indicated below. *B*, Western blot analysis shows that rat anti-Homer antibodies specifically recognize a 47 kDa band in protein extracts from a single adult fly head (closed arrowhead). Protein extracts were loaded as follows: Canton-S (lane 1), EP(2)2141 (lane 2), *homer*^{R102} (lane 3), and C155-GAL4/+; UAS-HomerΔC/+ (lane 4). Expression of HomerΔC in the CNS with the C155-GAL4 driver results in an additional band of ~27 kDa (open arrowhead). Anti-Discs-large (*DLG*) antibodies were used as a loading control. *C*, *In situ* hybridization to whole-mount late stage 16 embryo reveals that *homer* mRNA is highly expressed in the CNS (arrowhead points to ventral nerve cord). *D*, *In situ* hybridization to a dissected stage 16 embryo shows that Homer is expressed by most if not all neurons in the ventral nerve cord. *E*, *homer* mRNA is expressed in the embryonic PNS. Black arrowheads point to neurons of the chordotonal organ. Open arrowhead indicates the bipolar neuron. Scale bar: *C*, 100 μm; *D*, *E*, 16 μm.

full-length DIG-labeled *homer* antisense probe to *Drosophila* embryos. We detected a widespread, low-level expression of *homer* during early embryogenesis. Beginning at stage 12 the levels of *homer* RNA increase in the developing CNS and PNS, such that by late stage 16, *homer* RNA is enriched in the nervous system (Fig. 1*C*). Close examination of *in situ* hybridization performed on dissected embryos shows that within the CNS and PNS of stage 16 embryos, most if not all neurons express *homer* RNA (Fig. 1*D,E*).

Homer protein is concentrated in the neuropil

We raised a polyclonal antibody against Homer (see Materials and Methods) that recognizes a 47 kDa protein on a Western blot of protein extracts from adult fly heads (Fig. 1*B*). This size is in agreement with the protein predicted from the sequence of *homer*

cDNAs. Two results confirm the specificity of the anti-Homer antibody. First, no signal can be detected in protein extracts of flies homozygous for a *homer* loss-of-function allele (see below) (Fig. 1*B*, lane 3). Second, the antibody specifically recognizes a truncated version of Homer lacking the C terminus (HomerΔC) in protein extracts of flies that express HomerΔC in the CNS from a transgene (Fig. 1*B*, lane 4).

Using the anti-Homer antibody, we performed immunofluorescence staining of *Drosophila* embryos (Fig. 2). In wild-type embryos, we detected high levels of Homer expression in the CNS (Fig. 2*A*) and PNS (Fig. 2*G*), with a low level of expression detectable in other tissues, including the epidermis, the gut, and the somatic muscles. As expected, we found all staining to be abolished in embryos homozygous for the *homer*^{R102} mutation (Fig. 2*D*). Homer expression in the nervous system is maintained throughout development into adulthood, where in the brain it is expressed at high levels within the lamina and the medulla neuropil of the optic lobes and at lower levels in the central brain neuropil (data not shown).

Within the embryonic ventral nerve cord (VNC), the majority of staining is concentrated in the neuropil regions of each neuromere (Fig. 3*B*). Confocal analysis of immunostainings using anti-Homer and antibodies to HRP, which recognize a neuronal surface epitope present on all axons and dendrites (Jan and Jan, 1982), reveals that Homer is highly enriched in the dorsal-most region of the neuropil (Fig. 3*A,B,G*). To examine whether this enrichment might represent localization of Homer to synaptic regions, we performed triple labeling for anti-HRP, Homer, and the synaptic protein Synaptotagmin. We found that Synaptotagmin is similarly localized to the dorsal region of the neuropil (Fig. 3*C*) and that Homer extensively colocalizes with it (Fig. 3*I*). These results suggest that in *Drosophila*, Homer is targeted to synapses, similar to the localization of vertebrate Homer proteins (Brakeman et al., 1997; Xiao et al., 1998).

Homer colocalizes with BiP, a marker of the endoplasmic reticulum

A striking feature of Homer expression is the punctate pattern seen in neuronal cell bodies (Fig. 2*A,G*), a pattern reminiscent of the staining of Golgi stacks (Stanley et al., 1997). By performing double immunostaining with anti-Homer and antibodies that label the Golgi (Stanley et al., 1997), we found that Homer labeling partially overlaps with the Golgi staining but does not fully colocalize with it (Fig. 4*D*, arrowheads). This suggested that Homer is localized in the closely juxtaposed ER. To label the ER compartment, we used a monoclonal antibody generated against the rat ER-resident protein BiP (Huovila et al., 1992) that recognizes a *Drosophila* protein retained in the ER (Baumann, 2000). In double immunostainings we found that anti-Homer staining overlaps extensively with the ER marker anti-BiP, arguing for Homer localization in the ER compartment (Fig. 4*H*).

Mutation of the *Drosophila* Homer EVH1 domain abolishes binding to *Drosophila* Shank

Through the EVH1 domain, mammalian Homer-related proteins interact *in vivo* with at least three different group of proteins, Shank/ProSAP, Group I mGluRs, and the InsP3R (Brakeman et al., 1997; Tu et al., 1998; Naisbitt et al., 1999; Tu et al., 1999). To determine whether such interactions might exist in *Drosophila*, we constructed a yeast two-hybrid bait consisting of the Homer EVH1 domain fused to the DNA-binding domain of the GAL4 transcription factor. We also created a mutated Homer EVH1

Figure 2. Localization of Homer protein in the nervous system. *A–C*, Confocal images of the ventral nerve cord of a stage 16 wild-type embryo stained with anti-Homer (*A*), anti-HRP (*B*) antibodies, and the merge (*C*). Staining is concentrated in the ladder-like neuropil (*A*, arrowhead) containing neuronal processes that stain with anti-HRP. Punctate staining is detected in neuronal cell bodies (*A*, arrowhead). *D–F*, Confocal images of the ventral nerve cord of a stage 16 *homer* mutant embryo stained with anti-Homer (*D*) and anti-HRP (*E*) antibodies and the merge (*F*). Anti-Homer staining is totally abolished in *homer* mutant embryos. *G–I*, Confocal images of the PNS chordotonal organ in a wild-type embryo stained with anti-Homer (*G*) and mAb 22C10 (*H*) antibodies and the merge (*I*). PNS neurons were visualized with the mAb 22C10 antibody, which recognizes the microtubule-associated protein Futsch, expressed by most neurons of the *Drosophila* nervous system (Hummel et al., 2000). Note the punctate distribution of Homer in the neuronal cell bodies of the PNS chordotonal organ (*G*, arrowheads) and the somatic muscles (*G*, arrow). Scale bar, 10 μ m.

Figure 3. Homer colocalizes with the synaptic marker Synaptotagmin in the dorsal-most region of the neuropil. *A–L*, Confocal images of the ventral nerve cord of a stage 16 wild-type embryo triple-labeled with anti-HRP (*A*), anti-Homer (*B*), and anti-Synaptotagmin (*C*) antibodies. *A–C* and *G–I* show cross sections through a Z-series of confocal images (dorsal is up and ventral is down) at the level indicated by the white line in *D*. *D–F* and *J–L* show the Z-series confocal image corresponding to the optical section denoted by the white line in *A*. The extent of the neuropil is visualized with anti-HRP (*A*). Homer and Synaptotagmin staining are concentrated in the dorsal-most part of the neuropil (*B*, *C*, arrows) and extensively overlap, suggesting that Homer is localized to regions of the neuropil enriched in synapses. Scale bar, 10 μ m.

bait (mutEVH) by introducing several amino acid substitutions in the EVH1 domain (Fig. 5A). Noteworthy is the substitution of the second glycine in the conserved GLGF motif for a histidine. The crystal structure of the vertebrate Homer-1 EVH1 domain bound to its binding-peptide has shown that any residue other than

glycine at this position (position 89 of Homer-1) would be likely to sterically interfere with binding of Homer to its partners (Beneken et al., 2000).

From a yeast two-hybrid screen of a *Drosophila* embryonic library using the Homer EVH1 domain as bait, we isolated the single fly ortholog of the vertebrate Shank proteins (see Materials and Methods). Like the vertebrate Shank proteins, *Drosophila* Shank contains the Homer-binding consensus motif PPxxF near its C terminus. As shown in Table 1, the Homer EVH1 domain shows interaction with the C terminus of *Drosophila* Shank in the yeast two-hybrid assay. In contrast to the wild-type Homer EVH1 domain, mutEVH fails to interact with Shank C terminus, consistent with the introduced EVH1 mutations abolishing binding. As expected, neither the Homer EVH1 domain nor mutEVH showed any interaction with the C terminus of the characterized *Drosophila* Group II DmGluRA (Parmentier et al., 1996), which like vertebrate Group II mGluRs lacks any Homer binding site consensus sequence. These results argue that the ability of Homer to bind Shank is dependent on an intact EVH1 domain and further suggest that at least one of the protein–protein interactions mediated by the EVH1 domain of Homer-related proteins has been evolutionarily conserved.

The subcellular localization of epitope-tagged Homer depends on the integrity of the EVH1 domain

The regional colocalization of Homer and Synaptotagmin dorsally in the VNC neuropil suggests that Homer is concentrated in synapse-rich regions. Because we detected little or no Homer in the axons of motor neurons and sensory neurons (Fig. 2A), it seemed possible that Homer might be localized to dendrites. To investigate this possibility we created an epitope-tagged version of Homer (Homer-myc) by fusing six c-myc epitopes to the C-terminal end of the protein (schematically represented in Fig. 5A). We also created Homer-myc-green fluorescent protein (GFP) in which six myc epitopes plus the GFP were similarly fused to the C terminus. The two epitope-tagged versions of Homer gave identical results in all the experiments described below. For expression we used the GAL4/UAS transactivation

Figure 4. Homer colocalizes with the endoplasmic reticulum marker BiP. *A–C*, Confocal images of the ventral nerve cord of a wild-type embryo stained with anti-Homer (*A*) and anti-Golgi (*B*) antibodies. *D*, Close-up view of the *white open box* shown in the merged image in *C*. Homer protein does not fully colocalize with, but is closely juxtaposed to, the Golgi marker (*D*, arrows). *E–G*, Confocal images of the ventral nerve cord of a wild-type embryo stained with anti-Homer (*E*) and the ER marker anti-BiP (*F*). *H*, Close-up view of the *white open box* shown in the merged image in *G*. Homer extensively colocalizes with anti-BiP staining, suggesting that Homer is localized in the ER (*H*, arrows). Scale bar, 10 μ m.

system (Brand and Perrimon, 1993). Homer-myc and Homer-myc-GFP were cloned downstream of the UAS regulatory sequences in the pUAS transformation vector, and multiple transformant lines were generated for each. To express Homer-myc in single neurons, we used the RCC-GAL4 line, which stochastically expresses GAL4 in a small subset of identified neurons, including aCC, pCC, and RP2 (Baines et al., 1999). When expressed in the aCC neuron using the RCC GAL4 driver, Homer-myc staining is detected primarily in the ipsilateral and contralateral dendritic arborizations (Fig. 5*B*), with little or no staining seen in the axon. This result indicates that Homer is primarily targeted to dendritic processes and thus might function postsynaptically.

To determine the importance of the EVH1 domain for Homer subcellular localization, we created mutEVH-Homer-myc by introducing in the EVH1 domain the same amino acid substitutions that disrupt binding of the Homer EVH1 domain to Shank (Fig. 5*A*). When expressed in the aCC neuron using the RCC-GAL4 driver, mutEVH-Homer-myc fails to label the dendrites and

Figure 5. Mutation of the EVH1 domain disrupts the subcellular localization of epitope-tagged Homer. *A*, Schematic representation of wild-type and mutated (*mutEVH*) Homer. The position of the RANTVYGLGF motif in the EVH1 domain is indicated. The amino acid substitutions introduced into mutEVH-Homer are indicated in red and include substitution of histidine for the glycine residue that is essential for Homer binding to the PPxxF motif (Beneken et al., 2000). *B*, *C*, The ventral nerve cord of embryos in which the aCC neurons stochastically express Homer-myc (*B*) or mutEVH-Homer-myc (*C*) using the RCC-GAL4 line. The embryos are stained with anti-myc antibodies followed by HRP immunohistochemistry. *B*, In an embryo expressing Homer-myc, high levels of myc staining are detected in the ipsilateral and contralateral dendrites of the aCC motor neuron (*white arrowheads*), whereas little or no staining is detected in the motor axons (*open arrowheads*). *C*, In contrast, mutEVH-Homer-myc does not label the dendrites of the aCC motor neuron, and the staining is restricted to the cell body (*arrowhead*). *D*, *E*, Confocal images of a dorsal cluster of PNS neurons stained with anti-myc antibodies in embryos expressing Homer-myc (*D*) or mutEVH-Homer-myc (*E*) using the pan-neuronal C155-GAL4 line. *D*, *Arrowheads* point to the punctate staining of Homer-myc in cell bodies of the dorsal cluster. In contrast, this punctate staining is abolished after mutation of the EVH1 domain (*E*). Scale bar: *B*, *C*, 16 μ m; *D*, *E*, 5 μ m.

instead remains in the cell body (Fig. 5, compare *B*, *C*). When expressed in the PNS using the pan-neuronal C155 GAL4 driver, Homer-myc has a punctate distribution in many neuronal cell bodies, similar to the pattern characteristic of endogenous Homer (Fig. 5*D*, compare with Fig. 2*G*). Double-immunofluorescence staining confirmed that in these cells, Homer-myc colocalizes with the ER compartment marker BiP (data not shown). In contrast to Homer-myc, mutEVH-Homer-myc fails to show the characteristic punctate ER distribution in PNS neurons and instead is evenly distributed throughout the cytoplasm (Fig. 5, compare *D*, *E*). Taken together, these results demonstrate that the EVH1 domain of Homer is required for its subcellular localization and further suggests that this localization may be mediated by one of the binding partners of Homer.

Table 1. The EVH1 domain of Homer is required for its interaction with *Drosophila* Shank

	Homer-GAL4DBD	Homer mutEVH-GAL4DBD	Media
Shank-GAL4AD	+	–	–Leu, –Trp +X- α -Gal
	+	–	–Leu, –Trp, –Ade
	+	–	–Leu, –Trp, –His
mGluRA-GAL4AD	–	–	–Leu, –Trp +X- α -Gal
	–	–	–Leu, –Trp, –Ade
	–	–	–Leu, –Trp, –His

The Homer-GAL4DBD and Homer mutEVH-GAL4DBD baits contain the first 186 amino acids of Homer fused to the GAL4 DNA binding domain. The Homer mutEVH-GAL4DBD bait has the same amino acids substitutions as those described in Figure 5A. The Shank-GAL4AD prey contains the last 114 amino acids of the *Drosophila* Shank fused to the GAL4 activation domain. The mGluRA-GAL4AD prey contains the cytosolic tail of the *Drosophila* mGluRA fused to the GAL4 activation domain. Positive interaction between bait and prey was detected by the ability to activate three different Gal4-dependent reporter genes allowing expression of the α -Gal enzyme (assayed on X- α -Gal supplemented media) and growth on media deprived of adenine (Ade) and histidine (His).

Generation of a *homer* mutation

In the Berkeley *Drosophila* Genome Project database we identified an EP line, EP(2)2141, in which a P element is inserted 60 bp upstream of the first exon of *homer*. Western blot analysis reveals that *homer* expression is not significantly reduced in flies homozygous for the EP(2)2141 insertion (Fig. 1B, lane 2). To create a mutation in the *homer* gene, we generated a deletion by excising the P element. We recovered a 1.5 kb deletion, *homer*^{R102}, which removes the first two exons and half of the third exon of the *homer* gene. Sequencing across the breakpoints of this deletion revealed that it removes the nucleotides coding for the first 168 amino acids of the Homer protein (Fig. 1A). The lack of staining on Western blots of protein extract from *homer*^{R102} mutants and immunofluorescence staining performed on *homer*^{R102} embryos confirmed the nature of the *homer*^{R102} allele (Figs. 1B, 2D). For use as a wild-type control, we also recovered a precise excision of the EP(2)2141 insertion. We confirmed the integrity of the *homer* locus in these flies by DNA sequencing across the P-element insertion site. We refer to this line as *homer*⁺. For all the experiments described below, the *homer*^{R102} and *homer*⁺ chromosomes were each placed in identical genetic backgrounds (see Materials and Methods).

homer^{R102} homozygous flies are viable and fertile. In addition, they do not display any obvious uncoordinated phenotype and are able to respond to visual stimuli that elicit the escape response (Thomas and Wyman, 1982). Thus, Homer does not appear to be required for general aspects of nervous system development, nor does it appear to play a critical role in basic synaptic transmission. Adult flies display no gross anatomical defects, and the overall organization of the nervous system is indistinguishable from wild type, as assessed with anti-HRP antibodies (data not shown). Moreover, we did not detect any defects in axon pathfinding during the development of the embryonic nervous system of *homer*^{R102} mutants as assayed with anti-Fasciclin II (mAb 1D4) and mAb 22C10 (Zipursky et al., 1984), both of which recognize discrete subsets of axons (van Vactor et al., 1993), and mAb BP102, which labels all CNS axons.

It has been reported recently that overexpression of Homer in the *Xenopus* developing nervous system results in aberrant axon pathfinding (Foa et al., 2001). Thus, we asked whether overexpression of *Drosophila* Homer throughout the developing CNS

would result in abnormal axonal pathfinding. We performed immunostaining of the ventral nerve cord of embryos carrying one copy of the pan-neuronal C155-GAL4 driver and one copy of the UAS:homer-myc transgene. We did not detect any pathfinding errors as assayed with anti-Fasciclin II, mAb 22C10, mAb BP102, and anti-HRP (data not shown). Together, these loss- and gain-of-function data strongly suggest that Homer does not play a major axon guidance role in the *Drosophila* embryonic nervous system.

homer^{R102} mutant males show behavioral plasticity deficits in courtship conditioning

It has been proposed that in vertebrate, Homer-related proteins might regulate synaptic plasticity possibly underlying learning and memory via the modulation of mGluR function (Xiao et al., 2000). To test for a possible role of Homer in behavioral plasticity, we evaluated the performance of *homer*^{R102} mutant males in a courtship conditioning assay, an associative learning paradigm in *Drosophila* (Kamyshev et al., 1999). Courtship is a plastic behavior that can be conditioned by previous experience (Siegel and Hall, 1979; Tompkins et al., 1983; Greenspan and Ferveur, 2000). Male flies display a complex and robust courtship behavior toward a female in response to olfactory, visual, and tactile cues. After exposure to a nonreceptive mated female, wild-type males will repress their level of courtship. This repression is sustained during subsequent exposure to a receptive, virgin female. Courtship repression is thus a conditioned behavior and is thought to depend on the association of positive stimuli with an aversive chemosensory signal from the mated female (Tompkins et al., 1983).

In the courtship conditioning assay, individual males were placed with a nonreceptive mated female in a conditioning chamber for 1 hr. The mated female was then removed, and after 2 min, each male was individually tested for levels of courtship with an anesthetized virgin female (see Materials and Methods for details). The amount of courtship displayed by these “trained” males was monitored over a 10 min period and compared with courtship levels of “naive” males that had been manipulated identically in the conditioning protocol except without the nonreceptive mated female. In the assay, we did not detect any defects in the sequence or the length of the different steps of male courtship behavior of *homer* mutants, and thus *homer* is not essential for the execution of the courtship behavior.

Figure 6A shows the results of the courtship conditioning assay performed on *homer*^{R102} mutant flies and *homer*⁺ controls. Because it has previously been argued that courtship measurements from such conditioning assays are not normally distributed (Tompkins et al., 1983; van Swinderen and Hall, 1995; Kane et al., 1997; Kamyshev et al., 1999), we present the results of these experiments as raw data instead of means with SEs. For each group, the amount of courtship displayed by each individual fly is plotted, and the median values are denoted. Median values for naive and trained *homer*⁺ control males are 346 and 74 sec, respectively. For *homer*^{R102} mutants, median values are 437 sec (naive) and 335 sec (trained). As expected, *homer*⁺ control males show a statistically significant reduction of courtship after training ($p < 0.05$; multiple comparisons Dunn rank sums test), as do *homer*^{R102}/*homer*⁺ heterozygotes ($p < 0.05$; $n = 5$). In contrast, *homer*^{R102} trained males show no significant reduction of courtship ($p > 0.05$). In addition, although there is no significant difference in the amount of courtship displayed by naive *homer*^{R102} and naive

Figure 6. *homer* mutants show higher courtship levels and behavioral plasticity deficits in courtship conditioning assays. *A*, The courtship levels of individual *homer*⁺ wild-type males (closed gray diamonds) and *homer*^{R102} mutant males (open diamonds) toward an anesthetized virgin female are plotted. Courtship levels are defined as the amount of time spent courting over an observation period of 600 sec. For each genotype, trained males received the conditioning regimen with an unreceptive mated female, whereas naive males did not (see Results for details). A black bar indicates the median for each population. The *homer*⁺ wild-type males show normal conditioned suppression of their courtship after training. In contrast, *homer*^{R102} mutants fail to show conditioned suppression. Although the trained *homer*⁺ group is significantly different from each of the other three groups ($p < 0.05$; Dunn test), naive *homer*⁺, naive *homer*^{R102} mutants, and trained *homer*^{R102} mutants are not significantly different from one another ($p > 0.05$; Dunn test). *B*, The courtship levels displayed toward a mated female by *homer*⁺ wild-type males (closed gray diamonds) and *homer*^{R102} mutant males (open black diamonds) in the first (Initial) and last 10 min (Final) of the 1 hr conditioning period are plotted. A black bar indicates the median for each population. The *homer*^{R102} mutants show significantly higher levels of initial and final courtship when compared with *homer*⁺ ($p < 0.0001$ in a Mann–Whitney test). Nonetheless, *homer*^{R102} mutants significantly reduce courtship behavior after conditioning by the mated female ($p < 0.0005$ in a paired sign test).

homer⁺ flies ($p > 0.05$), the trained *homer*^{R102} mutant males show a significantly higher level of courtship when compared with the trained *homer*⁺ males ($p < 0.05$). Collectively, these results demonstrate that *homer*^{R102} mutants show behavioral plasticity deficits and fail to form and/or retain the conditioning by the nonreceptive mated female.

homer^{R102} mutant flies suppress courtship behavior during the conditioning but show higher courtship levels

To determine whether *homer*^{R102} mutants show defects in the acquisition of conditioning during training, we monitored the amount of courtship displayed by tested males over the first and last 10 min of the conditioning period (Griffith et al., 1994). Figure 6*B* shows the amounts of courtship displayed at the beginning (Initial) and end (Final) of the conditioning for both *homer*⁺ and *homer*^{R102} males. Two features of the distributions are evident from the graph. First, both *homer*^{R102} mutant flies and *homer*⁺ controls show a statistically significant reduction of courtship level after conditioning by the mated female ($p < 0.0005$ in a paired sign test). Second, *homer*^{R102} mutant males show higher initial and final courtship levels when compared with *homer*⁺ controls ($p < 0.0001$ in a Mann–Whitney test). Median values for initial and final courtship levels are, respectively, 331 and 133 sec for *homer*^{R102} mutants and 160 and 12 sec for the *homer*⁺ controls. These data demonstrate that *homer*^{R102} mutants do suppress

Table 2. Spontaneous locomotion and olfactory competence assays

Genotype	Spontaneous locomotor activity		Chemosensory jump response assay		
	Average number of line crossing	<i>n</i>	% CJR to propionic acid	% CJR to benzaldehyde	<i>n</i>
<i>homer</i> ^{R102}	86* ± 5	24	81 ± 3.5	97* ± 1	90
<i>homer</i> ⁺	64 ± 4	24	77 ± 1	83 ± 3	90

*Statistically different from *homer*⁺ controls ($p < 0.05$; Mann–Whitney test).

courtship behavior after conditioning by the mated female but show higher levels of initial and final courtship.

homer^{R102} mutant flies do not show olfactory defects but display deficits in the control of locomotor activity

It has been shown previously that the conditioned repression of male courtship is dependent on the perception of an aversive chemosensory cue secreted by the nonreceptive mated female (Siegel and Hall, 1979; Tompkins et al., 1983). Thus, defects in olfaction could be an explanation for the poor conditioning of *homer*^{R102} mutants in the courtship conditioning assay. To evaluate the olfactory competence of *homer*^{R102} adult flies, we used the chemosensory jump assay. When suddenly exposed to chemical vapors, a wild-type fly exhibits an escape response consisting of a jump (McKenna et al., 1989; Woodard et al., 1989). We tested the chemosensory jump response (CJR) of *homer* mutant flies to two different chemicals. The results of these experiments are shown in Table 2. The CJR to propionic acid of *homer*^{R102} mutant flies is similar to that of *homer*⁺ control flies, and the CJR of *homer*^{R102} mutant flies to benzaldehyde is actually higher than that of *homer*⁺ control flies, demonstrating that *homer*^{R102} mutant flies are not severely defective in olfaction. However, we found that *Homer* mutants do display deficits in their control of locomotor activity. *homer*^{R102} mutant flies show a higher level of spontaneous locomotor activity when compared with *homer*⁺ homozygous flies (Table 2), a feature that is consistent with *homer*^{R102} flies showing higher courtship levels.

DISCUSSION

homer is the single Homer-related gene in *Drosophila* and is expressed in the nervous system

In mammals, there are at least three independent genes encoding Homer-related proteins (Xiao et al., 1998). From searches of the *Drosophila* genomic sequence database we found that *homer* is the only fly gene encoding a Homer-related protein. The *homer* gene is expressed in a range of tissues, including all neurons within the nervous system. The mammalian Homer genes have partially overlapping expression patterns in the CNS and in non-neuronal tissues (Xiao et al., 1998). Thus, it is possible that in *Drosophila* the single widely expressed *homer* gene fulfills the functions of the multiple mammalian Homer genes.

Mammalian Homer 1a, the truncated Homer protein lacking the CC domain required for multimerization, is rapidly upregulated in multiple models of activity-dependent plasticity and is thought to modulate mGluR signaling by uncoupling mGluR–Homer–InsP3R complexes. We have no evidence for the existence of a fly counterpart to Homer 1a. Northern analysis reveals a single *homer* mRNA species, all cDNAs that we have analyzed encode full-length Homer, and Western analysis detects a band that corresponds to full-length Homer. Nonetheless, it is possible that we have not created the appropriate physiological conditions

for the expression of a truncated Homer and that such a truncated form might be induced by high levels of synaptic activity in flies.

Homer localizes to dendrites and the ER

The colocalization of Homer and Synaptotagmin in the dorsal-most region of the neuropil suggests that Homer is localized to regions containing a concentration of synapses. By expressing an epitope-tagged Homer, we provide evidence that the *Drosophila* Homer is targeted to dendrites, similar to the targeting described for vertebrate Homer proteins (Brakeman et al., 1997; Xiao et al., 1998; Tu et al., 1999). Within neuronal cell bodies, the *Drosophila* Homer colocalizes with a marker for the ER. In transfected cells, Homer 1b has similarly been shown to be localized in the ER compartment (Roche et al., 1999), a subcellular localization thought to be functionally relevant because Homer-related proteins are capable of binding the ER-resident receptor InsP3R (Tu et al., 1998). Thus, the evolutionary conservation of the subcellular localization of Homer-related proteins suggests that their functional roles might also have been conserved.

Homer binding to its putative partner(s) is required for ER and dendritic localization

Mammalian Homer binds to several proteins, including Shank, Group I mGluRs, and the InsP3 receptor (Tu et al., 1998; Xiao et al., 1998; Tu et al., 1999). Homer specifically binds to a short proline-rich motif, PPxxF, present in each of these proteins (Tu et al., 1998). In support of a functional role for this binding is the finding that mutations in the PPxxF motif of mGluR5 that abolish the binding to Homer *in vitro* also abolish the ER retention of mGluR5 in cells cotransfected with mGluR5 and Homer (Roche et al., 1999). In addition, mutation of the Homer binding site of mammalian Shank disrupts the Shank-dependent targeting of Homer 1b to dendritic spines (Sala et al., 2001). Here, we provide evidence that *Drosophila* Homer likely requires binding to at least one of its putative partners to be properly localized. Mutation of amino acids within the EVH1 domain predicted to be required for PPxxF binding abolishes the localization of Homer to the ER and its targeting to dendrites. Similar mislocalization has been described in cell culture for deletion of the EVH1 domain of Homer 2a (Shiraishi et al., 1999). At present, we do not know which protein regulates *Drosophila* Homer subcellular localization. Given our finding that the EVH1 domain of Homer binds *Drosophila* Shank, it is possible that Shank might have a function similar to what has been implicated in vertebrates (Sala et al., 2001). Further genetic and biochemical studies will be required to address this question.

In terms of candidate receptors in *Drosophila* to which Homer might bind, there are several putative mGluRs in the genome sequence database (Adams et al., 2000; Littleton and Ganetzky, 2000). Of these, only one, DmGluRA, has been characterized, and it has been pharmacologically classified as a group II mGluR (Parmentier et al., 1996). Consistent with this classification, the cytosolic domain of DmGluRA contains no Homer-binding motif and fails to bind Homer in our yeast two-hybrid assay. In vertebrates, two ER-resident proteins, the ryanodine receptor (RyR) and the InsP3R, both control intracellular Ca²⁺ stores and contain Homer-binding motifs in their cytosolically disposed N termini (Tu et al., 1998). Moreover, the InsP3R coimmunoprecipitates with Homer proteins (Tu et al., 1998). There is a single InsP3R and a single RyR in *Drosophila* (Yoshikawa et al., 1992; Adams et al., 2000; Sullivan et al., 2000), both of which contain putative Homer-binding motifs. It will be of interest to determine whether Homer binds *in vivo* to either of these receptors.

Homer function is required for the control of locomotor activity and behavioral plasticity

Our finding that *homer* mutant flies are able to walk, fly, and exhibit an escape response to visual stimuli suggests that Homer has no essential role in vision or the performance of basic motor skills. However, *homer* mutants are hyperactive for both spontaneous locomotion and courtship behavior, implicating Homer in the control of locomotor activity. The *homer* mutants also exhibit deficits in behavioral plasticity, as assayed in a courtship conditioning paradigm. In this type of assay, the *Drosophila* memory mutant *amnesiac* shows specific defects in the retention of courtship conditioning (Siegel and Hall, 1979). Similarly, *homer* mutants are capable of suppressing courtship behavior toward a nonreceptive mated female, but this suppression is not retained when subsequently tested with the virgin female. At present we cannot rule out the possibility that the defective control of locomotor activity interferes somehow with the formation and retention of the conditioning. Addressing this will require the genetic separation of *homer* function in the control of locomotor activity and behavioral plasticity.

In contrast to recent results suggesting a function for Homer-related proteins in axon pathfinding (Foa et al., 2001), we did not detect any obvious pathfinding defects in the CNS of *homer* mutant embryos. It is still possible, however, that *Drosophila* Homer could play a developmental role during synaptogenesis and that loss of Homer function results in structural defects undetectable at the light microscopy level used in our study. An alternative, but not mutually exclusive, possibility is that *Drosophila* Homer functions in the modulation of neuronal circuits by regulating synaptic plasticity, perhaps through the modulation of mGluR signaling. Loss-of-function of group I mGluRs in mice causes deficits in spatial learning and locomotor control (Aiba et al., 1994a,b; Conquet et al., 1994; Lu et al., 1997), and although the basic synaptic physiology in these mutant animals is unaffected, aspects of synaptic plasticity are impaired. Vertebrate Shank and Homer have also been implicated in the regulation of the structure and function of the synaptic junction (Sala et al., 2001). Our evidence for a physical interaction between *Drosophila* Homer and Shank raises the possibility of a similar synaptic function for these proteins in flies.

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