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A role for kinesin heavy chain in controlling vesicle transport into dendrites in Drosophila

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ABSTRACT The unique architecture of neurons requires the establishment and maintenance of polarity, which relies in part on microtubule-based transport to deliver essential cargo into dendrites. To test different models of differential motor protein regulation and to understand how different compartments in neurons are supplied with necessary functional proteins, we studied mechanisms of dendritic transport, using Drosophila as a model system. Our data suggest that dendritic targeting systems in Drosophila and mammals are evolutionarily conserved, since mammalian cargoes are moved into appropriate domains in Drosophila. In a genetic screen for mutants that mislocalize the dendritic marker human transferrin receptor (hTfR), we found that kinesin heavy chain (KHC) may function as a dendritic motor. Our analysis of dendritic and axonal phenotypes of KHC loss-of-function clones revealed a role for KHC in maintaining polarity of neurons, as well as ensuring proper axonal outgrowth. In addition we identified adenomatous polyposis coli 1 (APC1) as an interaction partner of KHC in controlling directed transport and modulating kinesin function in neurons.

INTRODUCTION

Neurons are highly polarized cells that contain two spatially and functionally distinct domains, the axonal and the somatodendritic domains. These domains maintain unique membrane structures and protein compositions and are essential for the neuron to receive and transmit signals. Disruption of this cellular organization can lead to loss of activity and neuronal death. While recent research sheds light on the mechanisms that control establishment of neuronal polarity during development and the involvement of microtubule-based motors, little is known about motor protein function and regulation during maintenance of polarity in differentiated neurons (Hirokawa and Takemura, 2005; Arimura and Kaibuchi, 2007).

Studies in cultured hippocampal neurons suggest a role of selective microtubule-based transport in the polarized distribution of at least a subset of dendritic proteins (Burack et al., 2000; Silverman et al., 2001). This seems to be a common mechanism for the localization of dendritic proteins, although the generality of this process remains to be proven. It has been suggested that so-called “smart” motor proteins that can distinguish between different subcellular destinations might mediate selective transport into dendrites (Burack et al., 2000; Goldstein and Yang, 2000; Shah and Goldstein, 2000). Three different hypotheses could explain how a smart motor can generate directed movement into dendrites. In the “structural hypothesis,” a smart motor could read structural differences between the axonal and dendritic cytoskeletons, such as composition of tubulin variants or posttranslational modifications (e.g., phosphorylation, acetylation or decoration with different microtubule-associated proteins [tau, MAP2]). Alternatively, in the “regulatory hypothesis,” a smart motor could read structural differences between the axonal and dendritic cytoskeletons. Studies in cultured hippocampal neurons suggest a role of selective microtubule-based transport in the polarized distribution of at least a subset of dendritic proteins (Burack et al., 2000; Silverman et al., 2001). This seems to be a common mechanism for the localization of dendritic proteins, although the generality of this process remains to be proven. It has been suggested that so-called “smart” motor proteins that can distinguish between different subcellular destinations might mediate selective transport into dendrites (Burack et al., 2000; Goldstein and Yang, 2000; Shah and Goldstein, 2000). Three different hypotheses could explain how a smart motor can generate directed movement into dendrites. In the “structural hypothesis,” a smart motor could read structural differences between the axonal and dendritic cytoskeletons, such as composition of tubulin variants or posttranslational modifications (e.g., phosphorylation, acetylation or decoration with different microtubule-associated proteins [tau, MAP2]). Alternatively, in the “regulatory hypothesis,” a smart motor could read structural differences between the axonal and dendritic cytoskeletons, such as composition of tubulin variants or posttranslational modifications (e.g., phosphorylation, acetylation or decoration with different microtubule-associated proteins [tau, MAP2]). 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We took an in vivo genetic approach to test these hypotheses for polarized protein transport in neurons. We examined the subcellular localization of the dendritic protein human transferrin receptor (hTfR) in Drosophila neurons and subsequently searched for mutants that interfere with its localization.

RESULTS

Human transferrin receptor localizes preferentially to dendrites in Drosophila neurons

A well-studied protein that localizes to dendrites in mouse hippocampal neurons via selective transport is hTfR (Burack et al., 2000). To explore whether this localization mechanism is evolutionarily conserved, we took advantage of the Gal4 system (Brand and Perrimon, 1993) and compared the subcellular expression pattern of a green fluorescent protein (GFP)-tagged version of hTfR in Drosophila neurons to the expression of cd8-GFP. Because cd8 is a transmembrane glycoprotein, its expression allows visualization of all cellular extensions (Lee and Luo, 1999). As in vertebrates, hTfR-GFP vesicles localize preferentially to dendrites in all neuronal cell types that we studied. GFP-positive vesicles can be found in dendrites but not distal axons in embryonic peripheral nervous system (PNS) and CNS neurons, compared with MAb 22C10, which stains all cellular processes in all PNS and some CNS neurons (Fujita et al., 1982; Figure 1, A–C). In 3rd instar larvae, hTfR-GFP vesicles are present in dendrites but fail to enter axons beyond the initial segment in PNS neurons (Figure 1E, arrowheads). In comparison, cd8-GFP can be found throughout the cell (Figure 1D, arrowheads). In addition,
Interestingly, when we expressed a hTfR-GFP construct that lacks the cytoplasmic domain, we observed strong localization to axons (Figure 1K, arrowheads) and dendrites (Figure 1J, arrows), similar to what was previously described in vertebrate tissue culture (West et al., 1997). In addition, the anti-GFP staining reveals a stronger localization of GFP to neurite membranes, as opposed to vesicular staining, which confirms the finding that the cytoplasmic domain of hTfR contains the dendritic targeting signal and the internalization signal (West et al., 1997).

We then compared hTfR localization to the subcellular distribution of an axonal marker, the kinesin family member kif21a (Marszalek et al., 1999). kif21a-GFP strongly localizes to axons in adult mushroom bodies (Figure 2, A–C), in contrast to hTfR-GFP, which is found in cell bodies, dendrites, and the axonal initial segment of mushroom-body neurons, while being excluded from distal axons (Figure 2, D–E).

**Mutations in the kinesin heavy chain gene mislocalize hTfR-GFP to axons**

To identify genes required for dendritic localization, we performed a loss-of-function screen to identify mutants in which hTfR-GFP mislocalizes to peripheral nerve axons in 3rd instar larvae. Initially, we screened a collection of 45 mutants in heterozygous condition for axonal appearance of hTfR-GFP in optic nerves and hTfR-GFP is largely excluded from photoreceptor axons (Figure 1G) and Bolwig’s nerve (Figure 1I), compared with cd8-GFP (Figure 1H, arrows).

GFP mislocalizes to peripheral nerve axons in 3rd instar larvae. In+/+, elavGal4/UAS-hTfR-GFP larvae, hTfR-GFP fails to enter segmental nerves (B) and CSP staining appears smooth (B′). (E–H) khc8/khc1ts; elavGal4/UAS-hTfR-GFP larvae. At 29°C, hTfR-GFP enters segmental nerves and forms organelle accumulations (F). Some of the GFP-positive clogs overlap with CSP-positive organelle accumulations (F′, arrow), others don’t (F, F′, asterisk). In addition, a mislocalization into optic nerves can be observed in the Khc mutants (G, arrows). (A and E) hTfR-GFP expression level in cell bodies of the ventral ganglion is comparable. In multidendritic cells of 3rd instar khc8/khc1ts larvae, a mislocalization of GFP into axons is evident (H, arrows) compared with wild type (D, arrows).
segmental nerves as candidates to test the structural hypothesis, the regulatory hypothesis, and the mixed polarity model (Table 1). None of the genes tested gave strong phenotypes under these conditions.

However, when we examined more carefully, we found that mutations in kinesin heavy chain (khc), the force-generating subunit of Drosophila kinesin-1 (Saxton et al., 1991), led to axonal accumulation of hTFR-GFP–positive vesicles. To overcome early larval lethality associated with khc loss-of-function alleles, we analyzed transheterozygous khc−/khc8-1ts82 alleles. When raised at the restrictive temperature of 29°C, khc8/khc1ts82 larvae survive to 3rd instar larval stages but exhibit a strong tail-flipping phenotype and accumulation of synaptic vesicle markers in segmental nerves (Saxton et al., 1991; Hurd and Saxton, 1996).

We observed obvious localization of hTFR-GFP in axons of segmental nerves, optic nerves, and multidendritic cells of khc8/khc1ts82 larvae (Figure 3, F–H). This aberrant localization is not caused by elevated expression levels of hTFR-GFP in mutants, since similar fluorescence intensity is observed in khc+/khc− and khc8/−/khc1ts82 mutant animals (Figure 3, A and E). No significant mislocalization into axons of hTFR-GFP could be observed in animals heterozygous for khc− (unpublished data).

We next tested whether complete loss of khc function affects the localization of other proteins in addition to hTFR-GFP. To exclude residual maternal contributions of Khc and to express hTFR-GFP in mutant cells, we generated loss-of-function neuroblast clones in mushroom bodies using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999). In wild-type clones, the distribution of hTFR-GFP was as expected, with strong accumulation in the axonal initial segment and most GFP vesicles being found in cell bodies and dendrites (Figure 4A). To label axons in mushroom bodies, we used mAb1D4 (anti-FasII). FasII immunoreactivity can be found in the axons of α and β lobes, and at reduced levels in the γ lobes. FasII is not ordinarily expressed in wild-type cell bodies and the calyx (dendrites; Figure 4A′; Crittenden et al., 1998). In khc clones, a range of phenotypes was observed. As expected, hTFR-GFP strongly mislocalized to and accumulated in axons beyond the initial segment (Figure 4, B–D). Severe neuronal morphogenetic defects were also uncovered using the FasII marker. Abnormal FasII accumulations were found in axons and pathfinding, and fasciculation and branching was disrupted, resulting in extra-long lobes, thinner lobes, or additional FasII-positive branches (Figure 4, B′–D′). In addition, we found mislocalization of FasII to the calyx and cell bodies (Figure 4D′). While the mislocalization of hTFR-GFP vesicles could be observed in all clones analyzed, the severity of the neuron morphogenetic phenotypes was more variable. We found different expression of fasciculation defects in 90% of the clones, but a clear ectopic expression of FasII specifically was observed in only 20% of the clones. Together, these phenotypes point toward a role of Khc in delivering guidance receptors, cell adhesion molecules, and other cargoes to their appropriate subcellular sites during development.

### Identification of APC1 as a potential khc-dependent dendritic localization factor in cytological location 98E3-F5

Our data suggest that Khc is required to transport hTFR-GFP into dendrites. We next asked what might regulate directionality of Khc movement. To set up a modifier screen, we took advantage of the fact that a 50% reduction in Khc does not lead to mislocalization of the hTFR-GFP marker, whereas a more pronounced protein reduction in the khc+/khc1ts82 animals results in axonal entry of hTFR-GFP. After screening 50 third-chromosomal deficiencies for enhancers of Khc, we recovered eight lines that, when transheterozygous with khc−/khc8-1ts82 animals results in axonal accumulation of hTFR-GFP. After screening 50 third-chromosomal deficiencies for enhancers of Khc, we recovered eight lines that, when transheterozygous with khc−/khc8-1ts82 animals results in axonal entry of hTFR-GFP. After screening 50 third-chromosomal deficiencies for enhancers of Khc, we recovered eight lines that, when transheterozygous with khc−/khc8-1ts82 animals results in axonal entry of hTFR-GFP. After screening 50 third-chromosomal deficiencies for enhancers of Khc, we recovered eight lines that, when transheterozygous with khc−/khc8-1ts82 animals results in axonal entry of hTFR-GFP. After screening 50 third-chromosomal deficiencies for enhancers of Khc, we recovered eight lines that, when transheterozygous with khc−/khc8-1ts82 animals results in axonal entry of hTFR-GFP.

| Structural proteins | Signaling/scaffolding | Motors |
|---------------------|-----------------------|-------|
| gammaTub37C         | Dsor                  | dhc   |
| alphaTub84B         | ksr                   | rob   |
| betaTub60D          | chrw                  | grid  |
| alphaTub67C         | phl                   | khc   |
| betaTub85D          | Ras85D                | Klp 64D |
| futsch K68          | rl (Df)               | KLP61F |
| futsch N94          | puc                   | KLP31E (Df) |
| futsch P28          | bsk                   | nod   |
| UAS-Map2            | syd                   | ncd   |
| UAS-Tau             | msn                   | neb   |
| Eb1                 | Ptp10D                | pav   |
| ctn                 | 14-3-2eta             |       |
| stau                | UAS-cdc42V12          |       |
| Gβ                  |                       |       |
| Gγ                  |                       |       |
| R(Rap1)             |                       |       |
| Rab5                |                       |       |
| Rab6                |                       |       |
| Rab8 (Df)           |                       |       |
| Rab11               |                       |       |
| Abl/TM6B            |                       |       |
| aPKC                |                       |       |
| Rho1                |                       |       |
| par-1               |                       |       |
| pka-C1              |                       |       |
| polo                |                       |       |
| AP-47 (Df)          |                       |       |
| ebi                 |                       |       |
| shi                 |                       |       |

**TABLE 1:** Candidate mutants screened for mislocalization of hTFR-GFP to axons in 3rd instar larvae.
pared them with a simple khc8/+ heterozygote or Df(3R)3450/+ heterozygote. (Figure 5, C–G). We found a 1.36-fold increase in mean pixel intensity in khc8/+; Df(3R)3450/+ transheterozygote animals over khc8/+; wt/+ and a 1.1- to 1.22-fold increase with two different apc1 alleles (khc8/+; apc1Q8/+ and kkh8/+; apc1×1/+; Figure 5H). In addition, when we calculated the cumulative pixel intensity distribution, we observed a statistically significant shift to higher pixel intensities in the khc8/+; apc1/+ transheterozygotes (Figure 5I). Thus the reduction of Apc1 enhances the phenotype of khc reduction and Apc1 and Khc may interact to control the transport of hTfR into dendrites.

Homozygous apc1Q8 animals survive to the 3rd instar larval stage at the restrictive temperature of 29°C. We therefore investigated whether we could observe a mislocalization of hTfR-GFP into axons in homozygous animals with wild-type amounts of Khc. Clear mislocalization of hTfR-GFP in optic nerves was found in homozygous apc1Q8 larvae compared with wt (Figure 6B). Quantification of mean pixel intensity in optic nerves revealed a 1.4-fold increase in apc1Q8 over wt (Figure 6C). In addition, since Apc1 is highly abundant in axons, we asked whether we could observe a transport defect in apc1 homozygous animals. We stained segmental nerves with cysteine string protein (CSP) antibodies (Zinsmaier et al., 1994) and observed accumulation of this synaptic vesicle marker in the nerves.

### TABLE 2: Third chromosomal deficiencies that lead to mislocalization of hTfR-GFP in a khc8/+ background.

| Degree mislocalization | Bloomington number | Name              | Breakpoints       |
|------------------------|--------------------|-------------------|-------------------|
| +++                    | 430                | Df(3R)3450        | 98E3; 99A6        |
| ++                     | 6754               | Df(3L)Fz2         | 75F10-11; 76A1-5  |
| ++                     | 6646               | Df(3L)BS20        | 76A7-B1; 76B4-5   |
| ++                     | 5126               | Df(3L)X5533       | 76B4; 77B1        |
| ++                     | 5694               | Df(3R)e1025-14    | 82F8-10; 83A1-3   |
| +                      | 3124               | Df(3L)Fz-GF3b     | 70C2; 70D5        |
| +                      | 3546               | Df(3R)BB1         | 99D3-3Rt          |
| +                      | 8103               | Df(3R)ED5177      | 83B4; 83B6        |
polarized transport are evolutionarily conserved. Using hTfR-GFP, we were able to take advantage of Drosophila genetics to test for the existence of a smart motor. In our loss-of-function screen for mutants that mislocalize the dendritic hTfR-GFP construct to axons, we identified a \( \text{khc} \) mutant that led to strong axonal accumulations of the hTfR-GFP. Interestingly, when we analyzed mutant \( \text{khc} \) clones in mushroom bodies, we observed a variety of neuron morphogenetic phenotypes, such as axonal pathfinding failures as well as defects in fasciculation and branching, in addition to the mislocalization of hTfR-GFP (Figure 4). Therefore, in addition to previously described roles for Khc in establishing the anterior–posterior axis in the oocyte and axonal transport of synaptic vesicles (Hurd and Saxton, 1996; Brendza et al., 2000),

**FIGURE 5:** Identification of APC1 as a potential Khc-dependent dendritic localization factor. (A) UAS-hTfR-GFP, \( \text{khc}8/\alpha \); \( \alpha \text{elavGal4}/+ \), (B) UAS-hTfR-GFP, \( \text{khc}8/\alpha \); \( \alpha \text{elavGal4/} \text{Df(3R)3450} \). Note the strong mislocalization of GFP to the optic nerve (B, arrow). Scale bar: 20 μm. (C) UAS-hTfR-GFP, \( \text{khc}8/\alpha \); \( \alpha \text{elavGal4/+} \), (D) UAS-hTfR-GFP, \( \text{khc}8/\alpha \); \( \alpha \text{elavGal4/} \text{Df(3R)3450} \), (E) UAS-hTfR-GFP, \( \text{khc}8/\alpha \); \( \alpha \text{elavGal4/} \text{apc}1^{Q8} \), (F) UAS-hTfR-GFP, \( \text{khc}8/\alpha \); \( \alpha \text{elavGal4/} \text{apc}1^{X1} \), (G) UAS-hTfR-GFP, \( \text{khc}8/\alpha \); \( \alpha \text{elavGal4/} \text{Df(3R)3450} \). Examples of optic nerves used for the quantification. Scale bar: 10 μm. (H) Note the increase in hTfR-GFP mean pixel intensity in optic nerves of the \( \text{khc8} \); APC1 transheterozygotes compared with the \( \text{khc8} \) heterozygotes. (I) The statistically significant left shift in the curves indicates a higher percentage of bright pixels in optic nerves of the \( \text{khc8} \); APC1 transheterozygote animals compared with the \( \text{khc8} \) heterozygotes (\( \text{khc8} \); Df(3R)3450 → \( p = 8.3405e-005; \text{X1} \rightarrow p = 2.1768e-005; \text{Q8} \rightarrow p = 0.0301, p \) values were obtained by running a Kolmogorow-Smirnov test).

of \( \text{apc}1^{Q8} \) larvae (Figure 6E). We quantified this phenotype and counted 2.47 clogs/mm nerve compared with 0.09 clogs/mm nerve in \( \text{yw} \) control larvae (Figure 6F).

**DISCUSSION**

In this study, we evaluated the involvement of microtubule-based transport in maintaining neuronal polarity and identified Khc as a motor that can mediate transport of cargo into dendrites. We established a model system in Drosophila by expressing a vesicular protein that is actively transported into dendrites, hTfR coupled to GFP (Burack et al., 2000). We found that the subcellular localization in dendrites of this vertebrate protein was mirrored in invertebrate neurons (Figure 1), which indicates the mechanisms that mediate its polarized transport are evolutionarily conserved. Using hTfR-GFP, we were able to take advantage of Drosophila genetics to test for the existence of a smart motor.

In our loss-of-function screen for mutants that mislocalize the dendritic hTfR-GFP construct to axons, we identified a \( \text{khc} \) mutant that led to strong axonal accumulations of the hTfR-GFP. Interestingly, when we analyzed mutant \( \text{khc} \) clones in mushroom bodies, we observed a variety of neuron morphogenetic phenotypes, such as axonal pathfinding failures as well as defects in fasciculation and branching, in addition to the mislocalization of hTfR-GFP (Figure 4). Therefore, in addition to previously described roles for Khc in establishing the anterior–posterior axis in the oocyte and axonal transport of synaptic vesicles (Hurd and Saxton, 1996; Brendza et al., 2000),
our data show that the kinesin motor is also needed throughout development to transport important guidance molecules to the growth cone and to carry dendritic cargo to its destination.

Khc is a plus end–directed motor that moves toward the plus ends of microtubules, which in axons are located toward the synapse. In vertebrates, dendrites have been shown to contain microtubules with mixed polarity (Baas et al., 1988), thus allowing plus-end motors such as kinesin to enter dendrites and axons. A recent study showed that proximal dendrites are equipped with 90% minus end–out microtubules in some Drosophila neurons (Stone et al., 2008), which would make kinesin an unlikely candidate to transport cargo into dendrites. However, in our study of khc loss-of-function phenotypes in segmental nerves, optic nerves, and mushroom bodies, we could show a mislocalization of the dendritic hTIR to axons (Figures 3 and 4). This clearly indicates that hTIR is a Khc cargo and suggests that Khc is able to actively transport hTIR into dendrites, despite a scarcity of plus end–out microtubules. Interestingly, we failed to observe an axonal mislocalization of hTIR-GFP in mutants of the minus end–directed dynein or dynactin motors (unpublished data), further supporting our model of hTIR-GFP being transported by a plus-end motor.

We suggest that Khc can be classified as a smart motor that actively travels in both axonal and dendritic compartments and, depending on its localization and/or cargo, must be differentially regulated. Alternatively, Khc could be part of a mechanism that excludes some dendritic proteins from falsely entering the axon. If Khc is able to enter various compartments and transports axonal as well as dendritic cargoes, how is its direction of travel determined? In a secondary modifier screen, we aimed to identify interaction partners of kinesin that control its localization and/or direction of travel. We found apc1, the Drosophila homologue of the tumor suppressor adenomatous polyposis coli (APC; Hayashi et al., 1997) to be a neuronal Khc modifier. Since o-APC or APC1 is a microtubule-binding protein and highly expressed in axons during development (Munemitsu et al., 1994; Smith et al., 1994; Hayashi et al., 1997; Ahmed et al., 1998), it was conceivable that it could interact with Khc in axons to prevent dendritic cargoes from being transported. In addition, it has been reported that APC colocalizes with Khc in epithelial cells and that disruption of Khc function abolishes peripheral APC localization (Cui et al., 2002), further supporting our finding of a possible collaboration between these two proteins in controlling directional transport.

Indeed, when we measured axonal appearance of hTIR-GFP in transheterozygote khc/apc1 animals, we found a significant increase in mislocalization of hTIR-GFP in optic nerve axons with several apc1 alleles and the deficiency when compared with khc8 heterozygote animals alone. Therefore APC1 cooperates with KHC in mediating dendritic restriction of hTIR. In addition, APC1 homozygous larvae that survive until the 3rd instar stages also show mislocalization of hTIR-GFP into axons, as well as a pronounced accumulation of synaptic vesicles in segmental nerves, thus revealing a previously unknown function for APC1 in transport in differentiated neurons. In developing hippocampal neurons, a similar role for vertebrate APC has been reported (Shi et al., 2004). In that study, the authors show that the polarity marker mPar3 is localized to the tip of growing axons via APC and kinesin-2–mediated transport and spatially regulated GSK-3β, confirming the tendency of APC proteins to associate with plus end–directed motors.

In summary, our data and the data of others reveal that mutations in both Khc and APC1 genes lead to defects in the localization of axonal and dendritic cargoes in differentiated Drosophila neurons. The data presented here allow us to propose the following model: in the wild-type neuron, axonally located APC1 prevents kinesin-1–bound hTIR from entering (Figure 7A). Therefore, in apc1

FIGURE 6: APC1 functions in transport of dendritic as well as axonal vesicles. (A and D) wt and (B and E) apc1Q8/ apc1Q8. A mislocalization of hTIR-GFP can be observed in the mutants compared with wt (B), as well as the appearance of CSP-positive organelle accumulations in segmental nerves (E, arrows). (C) Quantification of hTIR-GFP mean pixel intensity. (F) Quantification of CSP clogs.
homozygous mutants, kinesin-I aberrantly transports hTfR into axons (Figure 7B). In khc homozygous mutants, an unknown, generic motor protein binds to the dendritic cargo and randomly transports it into all compartments (Figure 7C). Similarly, in the transheterozygote khc/apc1 animals, axonal exclusion is no longer strong enough to prevent some dendritic hTfR bound to Khc from localizing into axons, and the decrease of a preferred motor allows some hTfR to bind to an unknown, generic motor (Figure 7D).

Taken together, our data strongly support the existence of a dynamic, smart motor that, dependent on its environment and cargo, can control the establishment and maintenance of neuronal polarity, thereby ensuring proper nervous system function.

MATERIALS AND METHODS

Drosophila stocks and genetics

Flies were raised on conventional medium at 25°C, and all fly work and recombination experiments were done according to common practice. apc1 stocks were obtained from Eric Wieschaus (Princeton University, Princeton, NJ). All other stocks were obtained from the Bloomington stock center (Bloomington, IN). The hTfR-GFP construct, a kind gift from M. Silverman and Gary Banker (Oregon Health Sciences University, Portland, OR), was subcloned into the pUAST vector, and transgenic flies were generated by P-element-mediated germline transformation.

Genetic mosaics were generated using the MARCM system (Lee and Luo, 1999) with FRT40, tub-Gal80 on the second chromosome and a elav-Gal4 driver on the third chromosome. To induce mitotic recombination in mushroom-body neuroblasts, staged larvae were heat-shocked for 30–40 min in a 37°C water bath 0–4 h after larval hatching, and then returned to 25°C until analysis.

Immunostaining

Larval segmental nerve immunostaining was performed as described previously (Hurd and Saxton, 1996). Immunostaining of embryos was done as described previously (Hummel et al., 1997). Immunostaining of wandering 3rd instar larval or adult nervous systems was done as follows: brains with attached segmental nerves and eye-antenna imaginal discs were dissected in phosphate-buffered saline (PBS; pH 7.2) and fixed with 4% formaldehyde for 20 min at room temperature. The samples were then rinsed and washed three times for 10–15 min each time in PBT (PBS, 0.3% Triton X-100) and blocked for 1 h in PBT plus 10% goat serum. Primary antibodies were incubated overnight at 4°C. The next day, samples were washed and incubated in secondary antibodies for 2 h at room temperature, washed again, and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

The following antibodies were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa: 22C10 (Fujita et al., 1982), anti-FasII: 1D4 (Goodman, unpublished data), and CSP (Zinsmaier et al., 1994). Anti-GFP (rabbit serum; Molecular Probes, Invitrogen, Carlsbad, CA), goat anti–mouse and goat anti–rabbit Alexa Fluor 488– and Alexa Fluor 594–coupled secondary antibodies were from Molecular Probes. All images were taken on an Olympus FV1000 spectral deconvolution confocal microscope (Olympus, Center Valley, PA) and processed using Image J (National Institutes of Health).

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