LETTER

Refining GAL4-Driven Transgene Expression in *Drosophila* With a GAL80 Enhancer-Trap

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Summary: We constructed an enhancer-trap element, P{GAL80}, that encodes the yeast GAL80 repressor to refine expression of transgenes driven by the binary GAL4/UAS system. GAL80 blocks GAL4 activity by binding to its transcriptional activation domain. We screened GAL80 enhancer-traps for repression of GAL4-induced green fluorescent protein (GFP) in the intact larval nervous system. We selected one line that repressed GFP in a large set of cholinergic neurons. This line was used to refine GFP expression from a set of over 200 neurons to a subset of 20 neurons in a preselected GAL4 line. Expression of tetanus neurotoxin, a potent blocker of neurotransmitter release, in these 20 neurons reproduced an aberrant larval turning behavior previously assigned to the parental set of 200 neurons. Our results suggest that targeted GAL80 expression could become a useful means of spatially refining transgene expression in *Drosophila*. genesis 39:240–245, 2004.
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In the fruit fly *Drosophila melanogaster*, tissue-specific transgene expression is achieved using the binary GAL4/UAS system (Brand and Perrimon, 1993). In this system, the yeast GAL4 protein is expressed in particular cells by using selected enhancers or promoters. GAL4 activates a gene of choice upon binding to the upstream activating sequence (UAS; Brand and Perrimon, 1993). Several collections of flies expressing GAL4 in selected cell types have been created both by mobilization of P-element transgenes encoding GAL4 to different genomic locations (e.g., Brand and Perrimon, 1993; Manseau *et al.*, 1997) or by fusing the coding sequence of GAL4 to promoter fragments (e.g., Luo *et al.*, 1994). A number of different UAS transgenes are available (for reviews, see Brand and Dormand, 1995; Phelps and Brand, 1998) that permit tissue-specific rescue of mutant phenotypes (e.g., Zars *et al.*, 2000), cell ablation (Zhou *et al.*, 1997), interference with specific cellular signaling pathways (Luo *et al.*, 1994), and suppression of synaptic activity

(Sweeney *et al.*, 1995) or neuronal excitability (White *et al.*, 2001).

In an effort to identify neurons involved in a stereotypic locomotor behavior of the *Drosophila* larva (Suster and Bate, 2002), we are using the GAL4/UAS system. One of the approaches we have taken is to search for GAL4 lines that label very small sets of neurons, so as to express neurotoxins to block their electrical activity and assay the consequences on behavior. So far we have found very few GAL4 strains that label small (<50), overlapping or homogeneous populations of neurons (M.L.S., unpubl. obs.). Instead, we find that most existing collections of GAL4 strains are expressed widely throughout the nervous system and in heterogeneous populations of cells. This is consistent with many reports of GAL4 expression patterns in the literature (e.g., Manseau *et al.*, 1997; Siegmund and Korge, 2001; Kido and Ito, 2002) and with the notion that most enhancers and promoters drive gene expression in multiple cell types and tissues simultaneously during development. Even GAL4 strains that label discrete populations of neurons, such as cholinergic (Kitamoto, 2001) or peptidergic (Siegmund and Korge, 2001) neurons, usually include hundreds, if not thousands, of cells. Currently, the only available method for targeting GAL4-driven transgene expression to single or small sets of related cells is the inducible FLP/FRT-recombinase system (Struhl and Bassler, 1993). This system, however, is of limited use for functional studies of the nervous system because GAL4 is expressed in different sets of randomly located cells in every animal. How does one then restrict
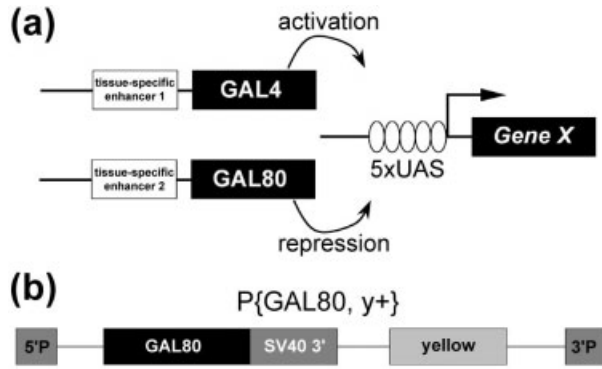
FIG. 1. The P{GAL80} enhancer-trap. **a:** Spatial refinement of GAL4-driven UAS transgene expression. GAL80 is under the control of a tissue-specific enhancer in a pattern that is different from but overlapping with that of GAL4. GAL4 activates a gene of choice through the upstream activating sequence (UAS) in defined cells. GAL80 represses GAL4 in some of these cells by masking its activation domain. **b:** pP{GAL80} vector. The GAL80 cDNA was fused to the 3′ terminus of the Simian Virus 40 (SV40). The 5′ and 3′ P-element inverted repeats are shown. The minimal coding sequence of yellow serves as a visible marker.

transgene expression to identical subsets of neurons in every animal? We adapted the mosaic GAL80 expression technique of Lee and Luo (1999) to spatially refine UAS transgene expression in *Drosophila*. We designed an enhancer-trap construct that encodes GAL80. We illustrate the use of this construct for subdividing the expression pattern of one preselected GAL4 line associated with discrete defects in larval locomotion.

In the GAL4/UAS system, GAL4 is expressed in defined sets of cells by means of a tissue-specific enhancer (Fig. 1a; Brand and Perrimon, 1993). We reasoned that by expressing GAL80 in flies using tissue-specific enhancers too, one could turn off GAL4 activity in subsets of GAL4-expressing cells (Fig. 1a). In *Drosophila*, ubiquitous expression of GAL80 with a tubulin promoter fully suppresses GAL4-driven pan-neural GFP expression (Lee and Luo, 1999). Repression of GAL4 activity by GAL80 is known to require the last 30 amino acids of GAL4 and results from blocking its activation domain through dimer–dimer interactions (Hirst *et al.*, 2001; Melcher and Xu, 2001). We thus modified the standard P{GAL4} enhancer-trap construct of Brand and Perrimon (1993) to express GAL80 in flies. The coding sequence of GAL4 was replaced with GAL80 (Fig. 1b). To follow the chromosomal location of P{GAL4} and P{GAL80} simultaneously in transgenic flies, the minimal coding sequence of *white* (a marker for eye color) in P{GAL4} was replaced by that of *yellow* (a marker for body color). Several independent P{GAL80} transgenic lines were generated that encode an ~49 kD protein consistent with the expected size of the GAL80 protein (data not shown; see Materials and Methods; Melcher and Xu, 2001).

We asked whether P{GAL80} could be used to repress GFP expression in the nervous system driven by GAL4 under a neural-specific promoter. As a first step, a simple screen was designed in which GAL80 lines were selected based on wide repression of GAL4-driven GFP fluorescence in cholinergic neurons (Fig. 2a). A P{GAL80} insert was mobilized by crossing to a source of transposase (Brand and Perrimon, 1993). Animals carrying GAL80 and transposase were crossed to flies carrying both a fusion of the choline acetyltransferase (cha) promoter to GAL4 and UAS-GFP. After screening ~2,000 larvae from this cross (all progeny) under a dissecting scope, we identified one line (28-3N-GAL80) that strongly repressed GFP expression in the central nervous system (CNS) and peripheral nervous system (PNS) (Fig. 2b,b′, respectively). Because we were only interested in GAL80 lines that repress GFP expression in large domains of the nervous system, we did not further study other lines that may have repressed GFP in small regions of the CNS.

To test the utility of the P{GAL80} enhancer-trap for refining sets of neurons involved in larval locomotor behavior, we studied 4C-GAL4 (Martin *et al.*, 2001). 4C-GAL4 is a typical example of an enhancer-trap widely expressed in the larval nervous system. When this line is crossed to a UAS line encoding a membrane-targeted mouse antigen CD8-GFP fusion (mCD8-GFP), a discrete population of over 200 neurons, including one sensory neuron, is consistently labeled in the larva from the first instar stage onwards (Fig. 3a), as well as salivary glands and fat body cells (data not shown). Using several other reporters, including UAS-tetanus toxin light chain (TeTxLC; Sweeney et al., 1995), we detect an essentially identical pattern of neurons labeled in the larval CNS (data not shown). When 28-3N-GAL80 is crossed to 4C-GAL4; UAS-mCD8-GFP we observe total suppression of GFP expression in the PNS and significant suppression (>180 neurons) in the CNS (Fig. 3b). In 4C-GAL4; UAS-mCD8-GFP/28-3N-GAL80 larvae (n > 30) a consistent set of lateral protocerebral (LP) neurons express GFP and are located in the anterior and dorsal part of the brain (LP1 and LP2 in Fig. 3a,b). In total, ~20 neurons are consistently labeled in these animals (n = 20 larvae) from the onset of GAL4 expression. The strong suppression of 4C-GAL4-driven GFP expression in the CNS and PNS is unlikely to be explained by a low ubiquitous expression of GAL80 from 28-3N-GAL80, as GFP fluorescence in nonneuronal tissues (e.g., fat body and salivary glands) of 4C-GAL4; UAS-mCD8-GFP is visibly unaffected by this GAL80 enhancer-trap.

We next asked whether 28-3N-GAL80 could repress a distinct locomotor phenotype linked to blockade of synaptic vesicle release in the 4C-GAL4 neurons by expression of tetanus toxin (TeTxLC; Sweeney *et al.*, 1995). This phenotype includes increased turning and reduced maximum speed. To do this we crossed 4C-GAL4 to UAS-TeTxLC (TNT-G); 28-3N-GAL80. Interestingly, 28-3N-GAL80 fully suppressed a speed deficit linked to 4C-GAL4/UAS-TNT-G but did not suppress aberrant turning when compared to the 4C-GAL4/UAS-TNT-VIF (inactive TeTxLC) control (Fig. 4a). The aberrant turning or increased speed of 4C-GAL4/UAS-TNT-G; 28-3N-GAL80 is
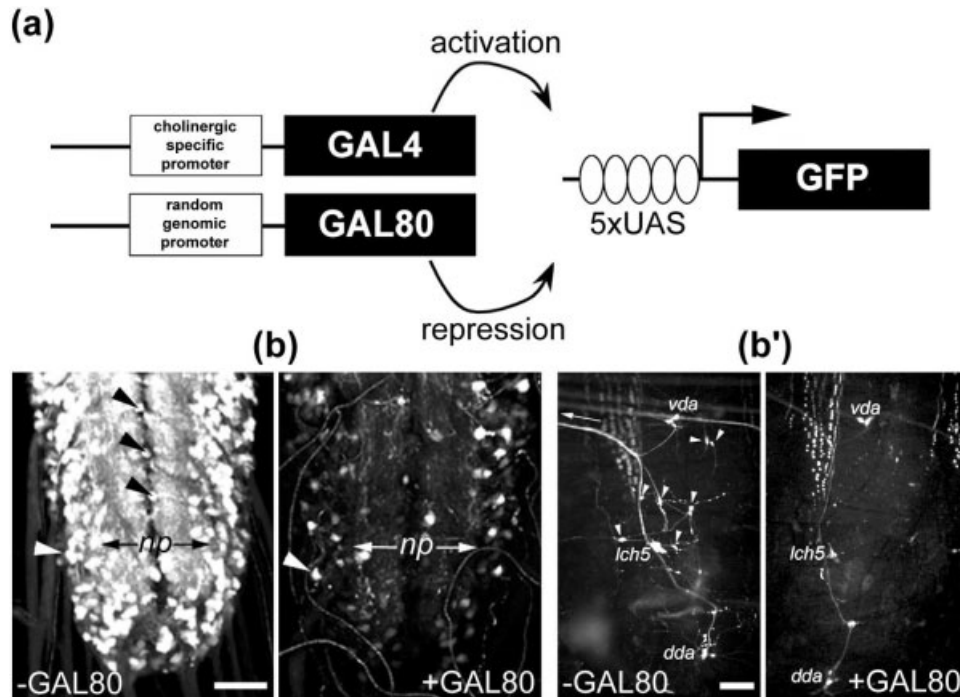
FIG. 2. Isolating P{GAL80} lines active in the nervous system. **a:** Strategy for identifying GAL80 lines. A GAL80 transgene was mobilized to "trap" random genomic enhancers that drive GAL80 expression strongly in the CNS, based on repression of GAL4-driven GFP expression in cholinergic neurons. **b:** A P{GAL80} enhancer-trap, 28-3N-GAL80, was obtained using the scheme shown in **a**. Images of GFP fluorescence in the ventral nerve cord (VNC) (**b**) or in one abdominal hemisegment (**b′**) of third instar cha-GAL4; UAS-GFP larvae with or without 28-3N-GAL80 (–GAL80 or +GAL80, respectively). Images in **b** were captured by laser confocal microscopy (3D projections) and those in **b′** (flat larval preparations) were captured using a light microscope equipped with GFP fluorescence filters. Anterior is to the top in **b**, and to the left in **b′** (white arrow in –GAL80 points towards the CNS). np and adjacent arrows in **b** indicate the extent of the neuropile. cha-GAL4, UAS-GFP/+ larvae (–GAL80) show strong GFP fluorescence in a large set of VNC neurons (**b**, –GAL80) including cell bodies near the dorsal midline (black arrowheads) and many located lateral to the np (white arrowhead). GFP expression is also detected in most sensory neurons of the PNS (**b′**, –GAL80), including the ventral dendritic arborization neurons (*vda*), lateral chordotonal organs (*lch5*), the dorsal dendritic arborization (*dda*) neurons (see Hartenstein, 1993) as well as in many other sensory neurons (white arrowheads). In contrast, cha-GAL4, UAS-GFP/+ larvae carrying one copy of 28-3N-GAL80 (+GAL80) show significant suppression of GFP expression throughout the VNC (**b**, +GAL80), although GFP expression remains virtually as strong in the soma of some neurons (compare cell labeled by white arrowhead in +GAL80 versus –GAL80). 28-3N-GAL80 almost completely suppressed GFP expression in the PNS (**b′**, +GAL80), except for a small number of sensory neurons (*vda, lch5, and dda*). Scale bar = 50.2 μm in **b** and 50 μm in **b′**.

not explained by the genetic background (*yw*) of the GAL80 insert, as animals homozygous for both *yw* and 28-3N-GAL80 display wildtype speeds (mean = 1.14 ± 0.06 versus 1.25 ± 0.05 mm/sec in 4C-GAL4/UAS-TNT-VIF control, $P > 0.05$) and turning rates (mean = 16.5 ± 2.8 versus 22.4 ± 2.2 deg/sec in control, $P > 0.05$, n > 18 per genotype). The fact that speed of 4C-GAL4/UAS-TNT-G larvae returns to normal after crossing to 28-3N-GAL80 suggests that the 180 neurons in which GAL4 activity is suppressed (Fig. 3b) may account for the speed deficit of these larvae.

One prediction from these results is that ubiquitous expression of GAL80 should further suppress both the speed and turning defects observed in 4C-GAL4; UAS-TNT-G larvae (Fig. 4a). To test this, we crossed 4C-GAL4, UAS-TNT-G, and transgenic flies that express GAL80 under the control of the tubulin promoter. tub-GAL80 strongly suppressed both the reduced speed (mean = 1.21 ± 0.06 mm/sec in +tub-GAL80, versus 0.61 ± 0.05 mm/sec in –GAL80) and turning rate defects (mean =

5.3 ± 0.2 deg/sec in +tub-GAL80 versus 33.9 ± 5.2 deg/sec in -GAL80) (for wildtype control, see Fig. 4a). Thus, our results show that GAL80 can be effectively used to either fully suppress or subdivide both the expression pattern and behavioral phenotypes of 4C-GAL4, a line that would otherwise not be amenable for further behavioral studies due to its widespread expression pattern.

Using GAL80 we have reduced the field of candidate neurons labeled by 4C-GAL4 that are associated with aberrant turning, from 200 to 20 neurons. Of these neurons, only one interneuron shows extended axonal projections in the VNC at the onset of GAL4 expression (#9 in Fig. 4b), while the rest (in the VNC) have projections that are either inconsistently labeled or too short to be traced. In the brain, five neurons with prominent axonal processes are consistently labeled in each hemisphere (#14–18 in Fig. 4b). Some of these neurons (LP1-2, cells #14–17) send projections to the ring gland and subesophageal ganglion (SEG), regions that play
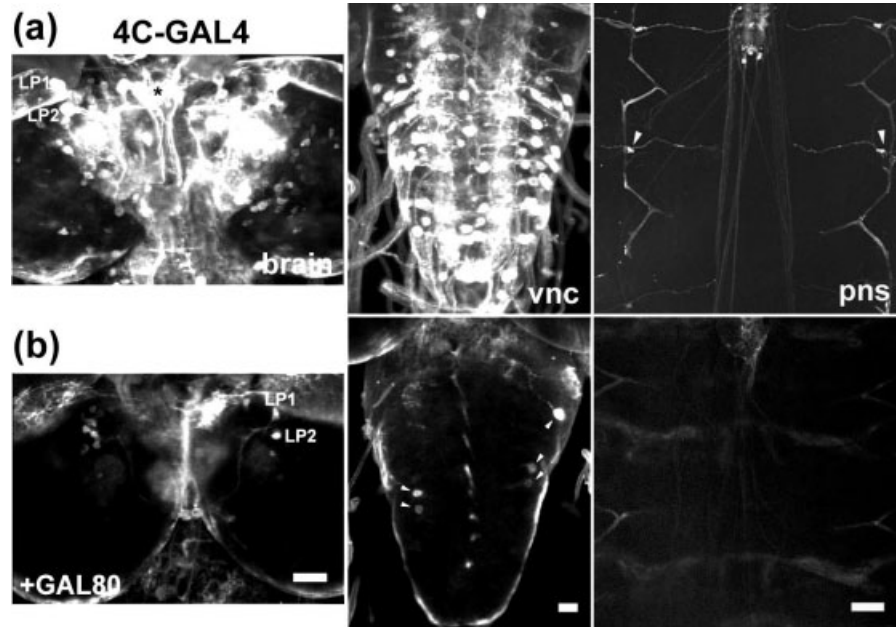
FIG. 3. Refinement of GFP expression driven by 4C-GAL4 with P{GAL80}.4C-GAL4 was crossed to UAS-mCD8-GFP and mCD8-GFP visualized in larval preparations using a mAb against mCD8. Images are 3D projections captured using a laser confocal microscope. **a:** 4C-GAL4 is expressed in population of over 200 neurons in the CNS including the brain and ventral nerve cord (vnc). Prominent lateral protocerebral cells (LP1-2) send projections to the ring gland (asterisk). A lateral bipolar dendrite sensory neuron (Hartenstein, 1993) is consistently detected in several hemisegments in the peripheral nervous system (pns). **b:** Upon crossing to 28-3N-GAL80, mCD8-GFP expression is detected only in the LP1-2 neurons and a few other cells in the brain. In the vnc, one neuron shows local axonal projections (top right half of vnc, marked by an arrowhead). A few other neurons are also labeled (arrowheads in lower half of vnc). No expression is detected in the pns. Scale bar = 32.9 μm for images of the brain, 23.5 μm for the vnc, and 65.8 μm for pns.
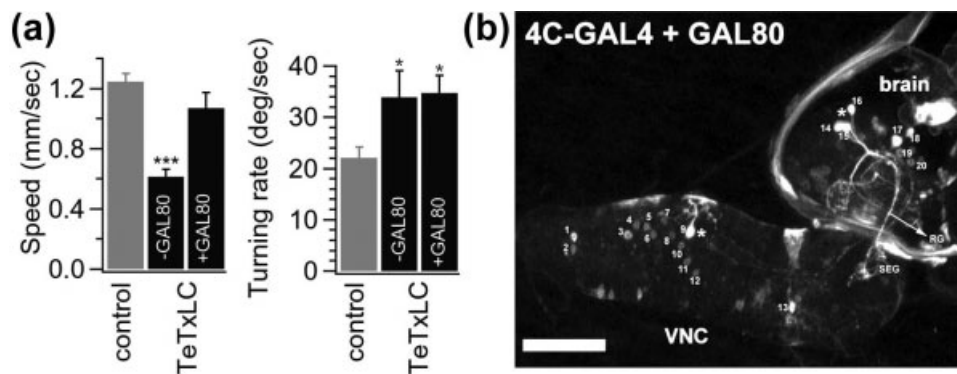
FIG. 4. Refinement of larval locomotor defects induced by TeTxLC in the 4C-GAL4 neurons with P{GAL80}. **a:** Parameters of larval locomotion in animals expressing TeTxLC in the original ~200 neurons of 4C-GAL4 or in the subset of 20 neurons revealed by 28-3N-GAL80. Maximum crawling speed of 4C-GAL4 is significantly reduced upon expression of TeTxLC in the original neurons (–GAL80, genotype: 4C-GAL4/UAS-TNT-G; n = 20) compared to wildtype animals expressing an inactive form of TeTxLC (control, genotype: 4C-GAL4/UAS-TNT-VIF; n = 19). 28-3N-GAL80 fully suppressed this speed deficit (+GAL80, genotype: 4C-GAL4/TNT-G; 28-3N-GAL80; n = 13). In contrast, a turning defect generated by expression of TeTxLC (–GAL80) in 4C-GAL4 is not suppressed by 28-3N-GAL80 (+GAL80). **$P < 0.001$, *$P < 0.05$ versus control (unpaired t-test). Error bars: ± SEM. **b:** 4C-GAL4 neurons revealed by GAL80 repression. A first instar CNS from a cross of 4C-GAL4; UAS-mCD8-GFP to 28-3N-GAL80 is shown laterally to visualize GFP fluorescence from ~20 neurons that persist to the third instar stage (shown in Fig. 3b). Posterior is to the left and dorsal to the top. Thirteen GFP-positive neurons (#1–13) are consistently observed in the VNC. Approx. 7 GFP-positive cells are found in the brain. Cell #9 has intensely labeled local projections in the VNC, while the rest have either faint or no detectable projections at this stage. Cells #14–17 include the LP1-2 neurons shown in Figure 3 with projections to the ring gland (RG) and subesophageal ganglion (SEG). Scale bar = 50 μm.

important roles in insect behavior, including locomotion (Burrows, 1996). Studies in *Manduca sexta* indicate that the SEG may play an important role in larval locomotion

(Dominick and Truman, 1986). It is possible that the neurons innervating the SEG (the LP1–2 neurons) could play a role in the modulation of larval turning in *Dro-*

sophila. In addition, cells #14–16 (Fig. 4b) have projections to the ring gland that are identical to those previously described as corazonin-expressing neurons in Siegmund and Korge (2001). Future studies using additional combinations of GAL4 and GAL80 enhancer-trap lines will be required to test the role of each of the 20 candidate neurons in larval locomotion.

GAL80 enhancer-trapping could become a useful means of refining GAL4-driven transgene expression in any tissue of choice. As shown here, new GAL80 enhancer-traps could be generated simply by screening for repression of GFP fluorescence in living tissues. This means that searching for GAL80 lines that repress GFP in cells that are readily visible through the cuticle (e.g., motoneuron or sensory neuron processes) should be feasible in large numbers. Alternatively, it may be beneficial to use a live marker such as GFP to positively label the cells that express GAL80. For developmental studies, P{GAL80} could provide a means of visualizing identical and small sets of GFP-labeled neurons that would otherwise be masked by dense GFP fluorescence in other neurons. In addition to P{GAL80} enhancer-traps, targeted expression of GAL80 with defined promoter fragments could be used to suppress transgene expression in specific cell types (Kitamoto, 2002). As the GAL4/UAS system becomes a practical reality in mice (Rowitch *et al.*, 1999), *Xenopus* (Hartley *et al.*, 2002), and zebrafish (Scheer and Campos Ortega, 1999), GAL80-mediated repression could also become a useful means of refining transgene expression in the vertebrate CNS.

MATERIALS AND METHODS

Fly Stocks

Stocks were maintained either in plastic bottles or vials containing a standard *Drosophila* medium (Ashburner, 1989) at $25 \pm 1°C$ and 60–80% humidity. All flies were maintained in a w^{1118} or *yw* background. Flies carrying a tubulin (tub) promoter-GAL80 fusion and UAS-mouse antigen CD8-GFP were obtained from the Bloomington Stock Center (Lee and Luo, 1999). P{UAS}-TeTxLC strains (UAS-TNT-G encoding active TeTxLC, and UAS-TNT-VIF encoding a catalytically inactive form of TeTxLC) were obtained from S. Sweeney. 4C-GAL4 has been previously described for the adult (Martin *et al.*, 2001).

Molecular Biology and Transformation of *Drosophila*

The pP{GAL80} enhancer-trap construct (Fig. 1b) was generated by modifying the PGawB vector (Brand and Perrimon, 1993) using standard molecular biology protocols (Sambrook *et al.*, 1989). The GAL4 coding sequence in PGawB was replaced with a 1.3 kb GAL80 cDNA (Lee and Luo, 1999) and the mini-$w+$ transgene replaced by a 5.2 kb $y+$ mini-gene (Patton *et al.*, 1992). This pP{GAL80, $y+$} construct was injected into a *yw* strain according to standard procedures. Two transformants were obtained on the third chromosome (GAL80-

28, GAL80-32) and one on the second chromosome (GAL80-14).

Protein Chemistry

Embryos were collected from transgenic flies overnight and homogenized using a protein extraction buffer (1.5%SDS/Tris-HCL, pH 6.8). Total protein from each extraction was subjected to SDS-PAGE and transferred by electrophoresis (Sambrook *et al.*, 1989) to nitrocellulose membranes. Immunodetection of GAL80 (49×10^3 Daltons, kD) was performed using an unpublished polyclonal Ab raised in rabbit against GAL80 (used at 1:1,000) kindly provided by Richard Reece (University of Manchester, UK) and signals visualized by chemical luminescence.

P{GAL80} Enhancer-Trap Screen

To generate new GAL80 enhancer-trap lines we mobilized a homozygous viable P{GAL80} insert (28-3) on the third chromosome by crossing to a source of P transposase on the X chromosome (Brand and Perrimon, 1993). Males carrying both P{GAL80} and transposase were then crossed to females carrying both a choline acetyltransferase (cha) promoter-GAL4 fusion and UAS-GFP on the second chromosome (Kitamoto, 2001). All larval progeny from this cross were screened live for changes in GFP fluorescence in the nervous system under a Wild Leitz (Canada) dissection microscope equipped with appropriate UV fluorescence filters (Biological Laboratory Equipment Maintenance and Services, Budapest, Hungary). Selected male larvae (not carrying the P transposase on the X) were then crossed to a *yw* balancer strain to remove the GAL4 and UAS transgenes.

Immunohistochemistry and Confocal Microscopy

Larvae were dissected along the dorsal midline (Keshishian *et al.*, 1993). For imaging GFP fluorescence, tissue was briefly fixed in 2% paraformaldehyde, washed, immersed in 60% glycerol for 5 min, then mounted under a glass coverslip. To detect mCD8-GFP, we used a rat mAb (CALTAG Labs, Burlingame, CA) diluted 1:50. Tissues were imaged using a Zeiss LSM 510 confocal microscope equipped with Kr/Ar/Ne lasers, except for those shown in Fig. 2b′, which were imaged using a Zeiss Axioscope under a $10\times$ objective with a Sony digital camera. Confocal sections were obtained at 2–5 μm intervals and reconstructed into 3D projections using Zeiss confocal software. Final images were assembled in Adobe PhotoShop (San Jose, CA).

Quantitation of Larval Locomotor Behavior

Larval crawling behavior was recorded at $23 \pm 2°C$ and $50 \pm 10\%$ humidity. Crawling patterns of 110–115-h-old (wandering) larvae were recorded using a 13 cm diameter 2.5% agar arena. The arena was placed on top of a cool-operating evenly illuminated fluorescent light box (Empix, Canada) and imaged using a CCD camera (a digital camera with a 1280×1024 pixel chip, Empix Imaging) equipped with a 55 mm macro lens (Nikon).

The camera lens, gain, and offset were adjusted so that the larva appeared as a distinct dark profile against a featureless, light background. Three-minute movies were captured at 1 frame per second (fps) using the Northern Elite image analysis system on a 1 GHz PC computer with FireWire technology (Empix Imaging) and analyzed off-line using the Dynamic Image Analysis System (DIAS), purchased from Solltech (Iowa, USA). Speed (mm/sec) and absolute turning rate (deg/sec) were obtained automatically from DIAS (Suster and Bate, 2002). Means were compared using unpaired *t*-tests in SPSS (Macintosh; Chicago, IL). Standard error of the mean (SEM) is used throughout the text and figures.

ACKNOWLEDGMENTS

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