Figure S1. (Related to main text, Fig. 1 and Fig. 2)

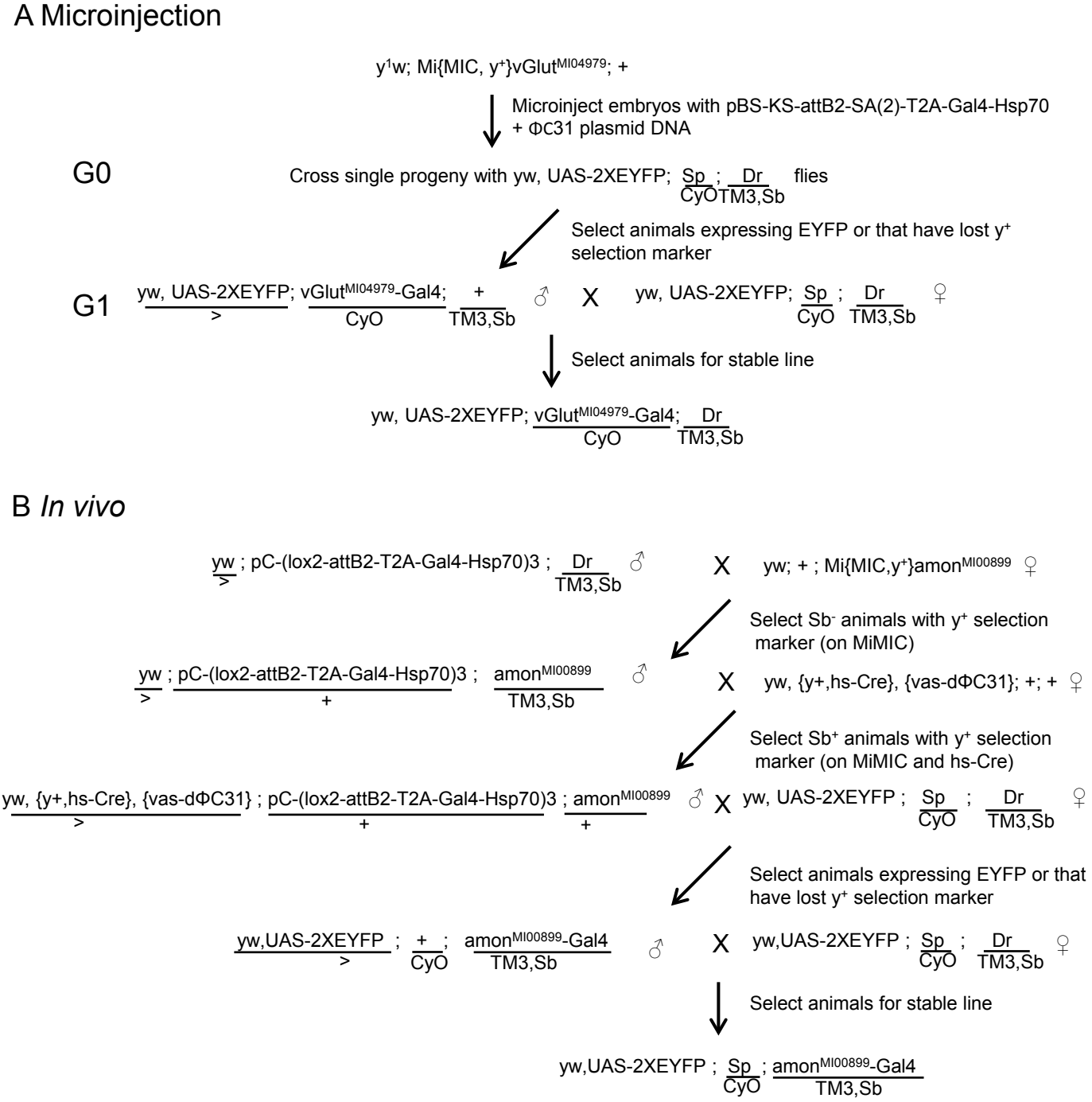

Figure S1. Methodology for generating Gal4 driver lines from MiMIC lines by embryonic microinjection of Trojan exon constructs or by using the "triplet donor" *in vivo* system
A) A Trojan exon plasmid (with the T2A-Gal4 sequence in the correct reading frame) is microinjected into embryos of a fly line bearing a MiMIC insertion (vGlut^{MI04979} in the example shown). ΦC31 (either co-injected or endogenously expressed) mediates cassette exchange in germ cells, which is accompanied by loss of the y$^+$ selection marker associated with MiMIC. Adults are crossed singly to flies of a "reporter-balancer line," and the progeny are screened by fluorescence microscopy. Adult

recombinants are selected to establish stable lines. In cases where fluorescence cannot be easily detected, yellow-bodied (y^-) progeny are selected and used to establish stable lines that can be analyzed by genomic PCR or by higher resolution fluorescence microscopy to identify those with T2A-Gal4 insertions. Variations on these methods can be used to identify recombinants for other Trojan exon constructs using the fly lines listed in Table S1. **B)** Using the *in vivo* triplet donor system requires bringing together several genetic components (accomplished as illustrated for the generation of the $amon^{MI00899}$- Gal4 line). First, the chromosome containing the MiMIC insertion is combined with the chromosome bearing the triplet donor (lines with the triplet donor on the X, II, and III are available; Table S1). Flies with both components are then crossed to flies from a “dual-recombinase” line, which has both germline-expressing Cre and ΦC31 transgenes on X. In the germline of the progeny of this cross, T2A-Gal4 sequences excised from the triplet donor by Cre form DNA circles, which serve as substrates for ΦC31-mediated cassette exchange into the MiMIC site. Adult progeny are then crossed to flies from the “reporter-balancer line” for screening as described above at (A).

Figure S2. (Related to Fig. 1B)

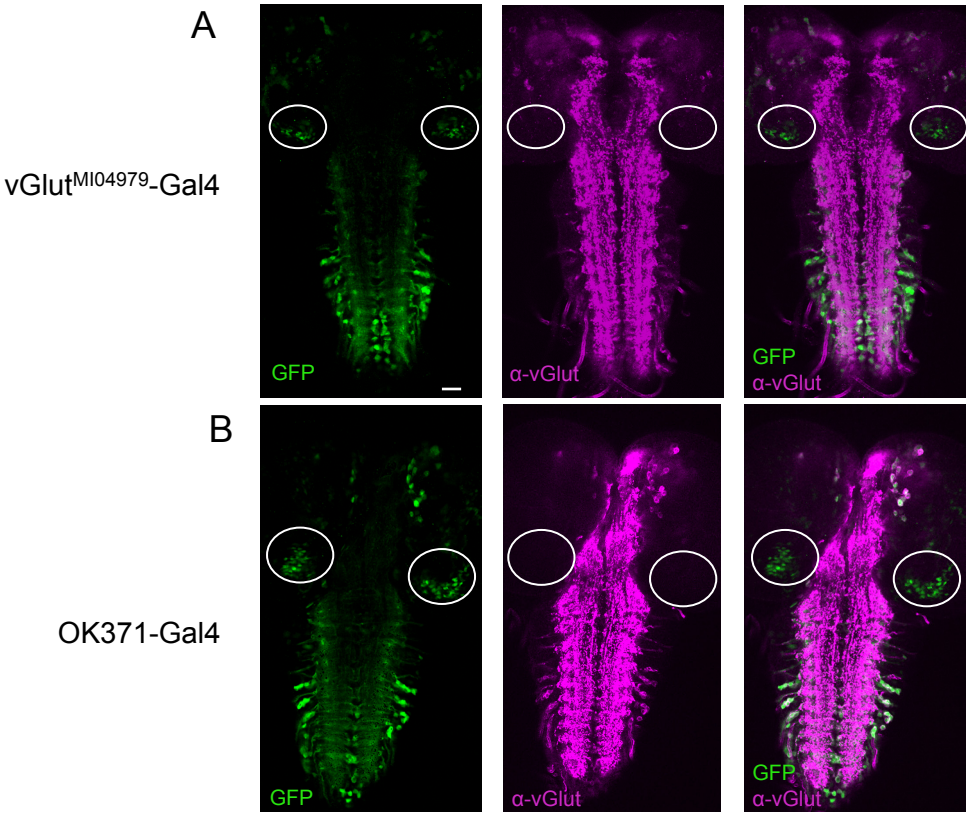

Figure S2. Glutamatergic drivers detect a subset of neurons not labeled by anti-vGlut antibody
A) Expression pattern of vGlut^{MI04979}- Gal4 line (left, UAS-EGFP) in the larval nervous system, double-labeled with anti-vGlut antibody (middle, magenta). The selected confocal planes show a set of neurons in the optic lobe (circle) that are within the vGlut^{MI04979}- Gal4 expression pattern, but are not immunopositive for the anti-vGlut antibody, as seen in the merged image (right). **B)** The expression pattern of the commonly used glutamatergic driver, OK371-Gal4, also labels the same set of optic lobe neurons. Scale bar, 25 μm.

Figure S3 (Related to Fig. 2A-B)

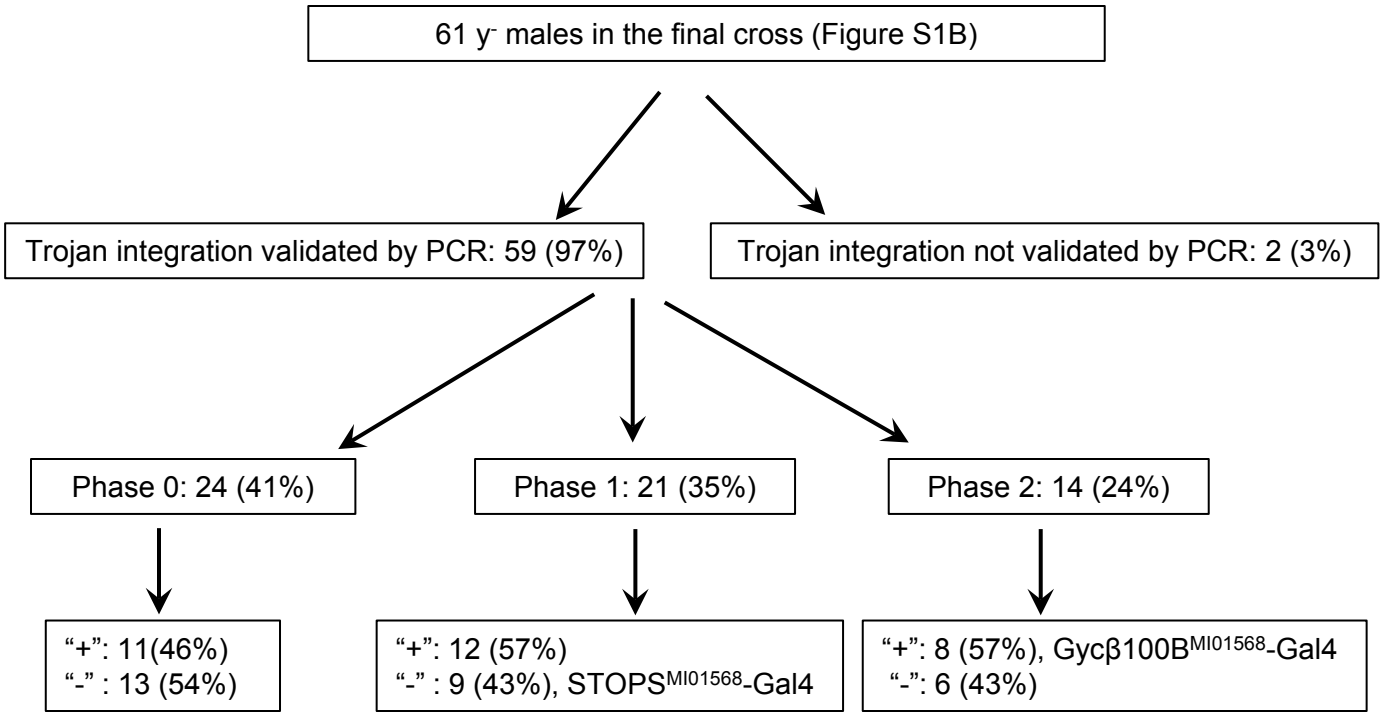

Figure S3. Analysis of the efficiency of cassette exchange of the three Trojan Gal4 donors into the MI01568 insertion into the *Gycβ100B* gene

To investigate the overall efficacy of Cre-mediated excision of the three Trojan Gal4 exons of the triplet donor and their ΦC31-mediated reintegration into the MI01568 MiMIC site, we established lines for 61 y^- progeny isolated solely on the basis of their loss of the yellow selection marker associated with the original MiMIC insertion (and without regard for their expression of a fluorescence reporter). We used PCR to first validate the successful incorporation of a Trojan exon into the MiMIC-associated attP landing sites (which occurred in 59 of the 61 cases) and then to verify the phases and orientations of the integrated Trojan exons. The number (and percentage) of lines having a given phase and orientation is indicated. "+" and "-" indicate orientation either in, or opposite to, the direction of transcription of the Gycβ100B gene, respectively. Little bias was observed for reintegration of any of the cassettes in the two possible orientations.

Figure S4 (Related to Fig. 2C)

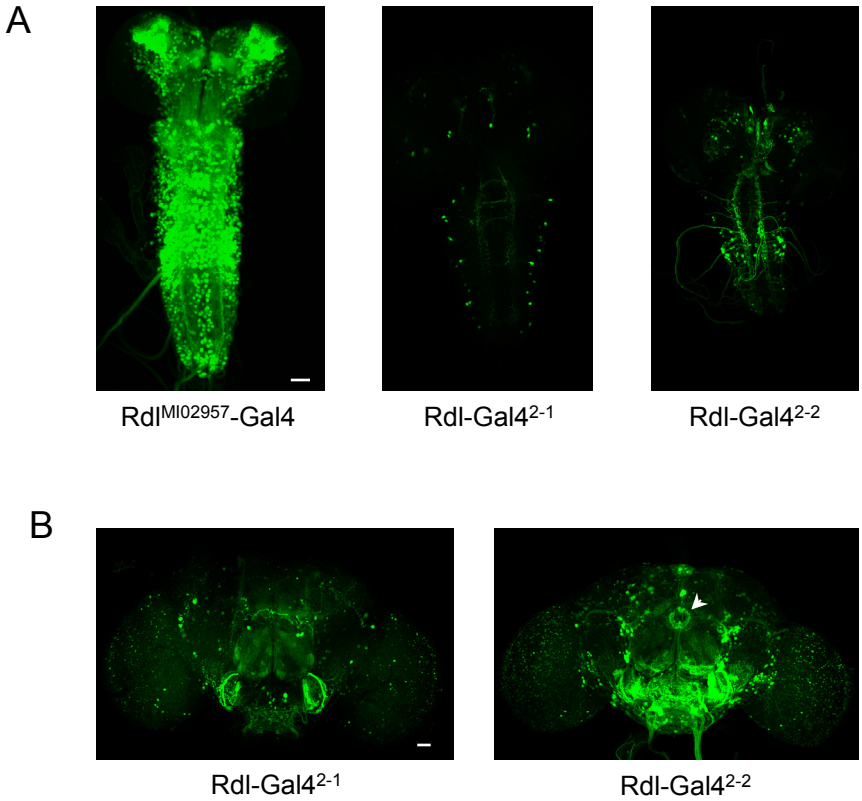

Figure S4. Comparison of Rdl^{MI02957}- Gal4 and two published Rdl-Gal4 drivers.
A) Expression patterns of Rdl^{MI02957}-Gal4 and two previously used Rdl-Gal4 driver lines visualized by UAS-EYP in larval CNS wholemounts. Left, Rdl^{MI02957}-Gal4; middle, Rdl-Gal4$^{2-2}$,, right, Rdl-Gal4$^{2-2}$. Images are volume-rendered confocal micrographs processed using the same acquisition parameters. Substantially fewer neurons are labeled in the preparations from the Rdl-Gal4$^{2-1}$ or Rdl-Gal4$^{2-2}$ driver lines. **B)** Rdl-Gal4$^{2-1}$ (left) and Rdl-Gal4$^{2-2}$ (right) expression patterns in adult brain wholemounts (green, UAS-EYFP). Adult brain structures known to strongly express the *Rdl* gene (see Fig. 2C"), are, in general, not prominently labeled by these drivers, with the exception of the Ellipsoid Body (arrow, right).Scale bars, 25 μm.

Figure S5 (Related to Fig. 2D)

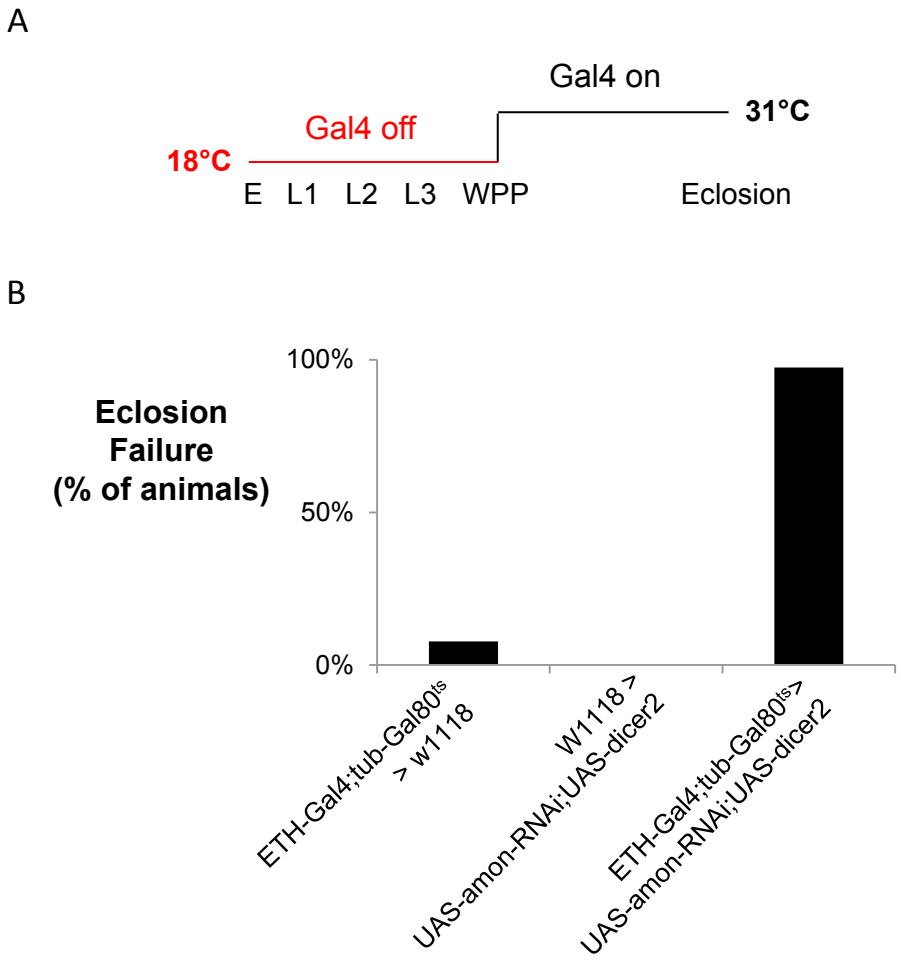

Figure S5. Knockdown of *amon* gene expression in Inka cells causes ecdysis deficits
ETH-Gal4 driven expression of UAS-amon-RNAi selectively in the Inka cells led to larval death and apparent ecdysis deficits. To better quantify the mortality, Gal80ts was used to temporally regulate the onset of UAS-amon-RNAi expression at the white prepupa stage (WPP) as diagrammed in (**A**) and the development of all animals was followed. **B**) The bar graph shows the percentage of animals that died of eclosion failure for two control genotypes that lacked either UAS-amon-RNAi or the ETH-Gal4 driver and for experimental animals expressing UAS-amon-RNAi and UAS-dicer2 under the control of the ETH-Gal4 driver.

Figure S6 (Related to Discussion, Fig. 1A, and Table 1)

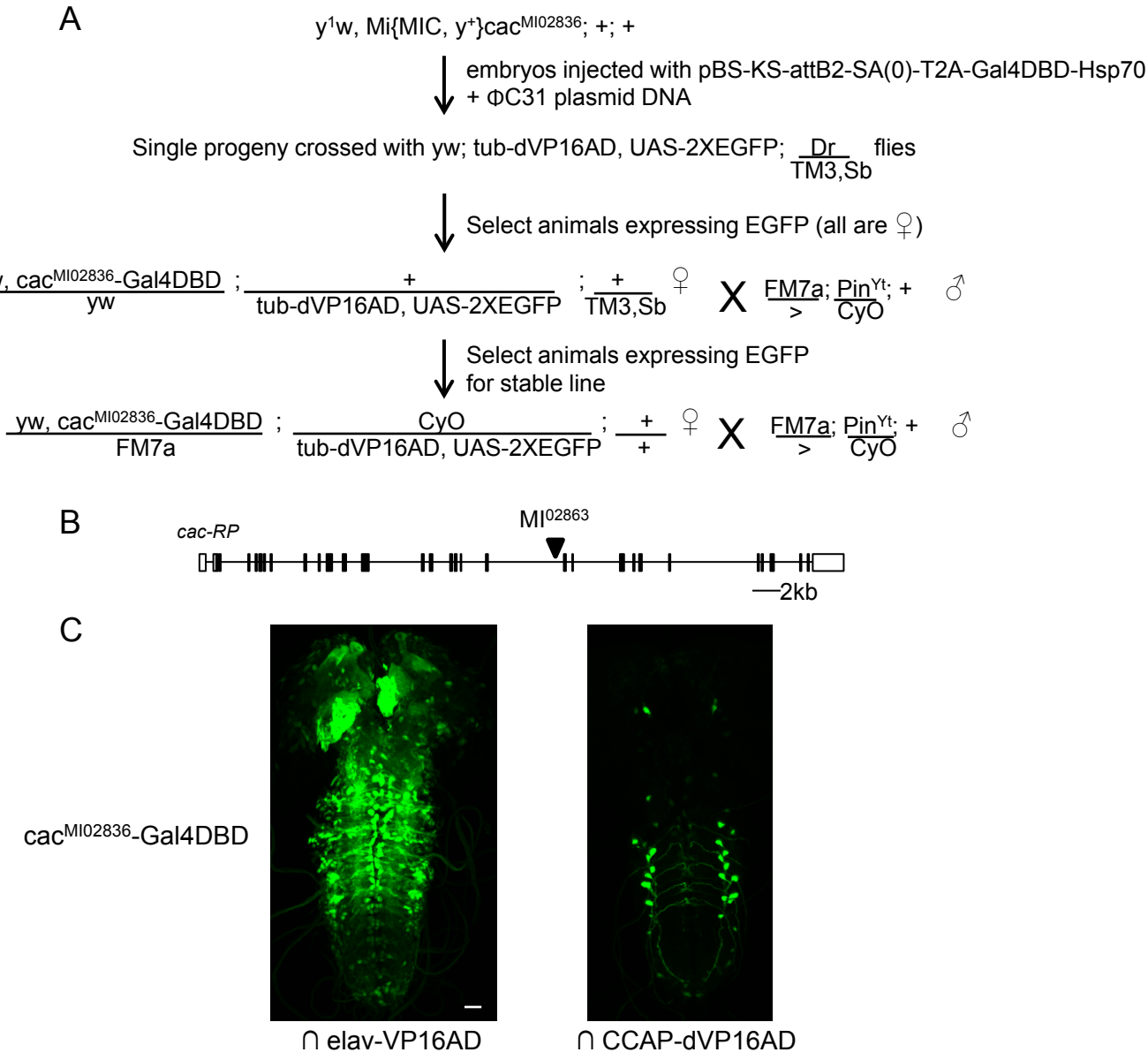

Figure S6. Generation of a Trojan-MiMIC line on X: cac^{MI02836}-Gal4DBD
MiMIC insertions into the coding introns of essential genes on the X chromosome require special consideration because truncation and loss of function will be lethal to male animals. **A)** Crossing scheme used to isolate and maintain a T2A-Gal4DBD insertion into the *cacophony* (*cac*) gene, which encodes an essential Ca^{++} channel a-subunit in *Drosophila*. This line can be maintained over the FM7a X-chromosome balancer, or alternatively by using an appropriate X-chromosome duplication. **B)** Schematic of the *cac* gene locus, showing the RP splice isoform and the MI02836 MiMIC insertion, which is located in an intron common to all splice isoforms. **C)** Confocal micrographs of the cac^{MI02836}-Gal4DBD expression pattern in the larval CNS, as revealed by crossing to the pan-neuronal elav-VP16AD hemidriver with UAS-EYFP as a reporter (left), or in the subset of neurons that express the neuropeptide CCAP, as revealed by crossing to a CCAP-dVP16AD hemidriver. The developmental lethality associated with the cac^{MI02836} insertion was rescued by an X chromosome duplication of the *cac* gene. Fly lines with duplications of X-linked genes have been placed onto chromosome III and are now available for almost every gene on the X-chromosome and can also be used to maintain lethal Trojan-MiMIC lines on X. Scale bar: 25 μm.

Table S1. Fly lines made to facilitate the generation of Trojan effector lines (Related to Experimental Procedures)

	Genotype of Line	Purpose of Line
Balancer Lines	yw; Sp/CyO; Dr/TM3,Sb	Balance Trojan inserts on II or III
	C(1)M3,y^2/>;Pin^{Yt}/CyO;+ x FM7a/>;Pin^{Yt}/CyO;+	Balance Trojan inserts on X
Lines for Screening Recombinants after ΦC31-mediated Cassette Exchange	yw,UAS-2XEYFP; Sp/Cyo; Dr/TM3,Sb	Screen Trojan Gal4 recombinants on II or III
	yw; UAS-2XEGFP; Dr/TM3,Sb	Screen Trojan Gal4 recombinants on X or III
	yw; Sp/Cyo; UAS-2XEGFP	Screen Trojan Gal4 recombinants on X or II
	yw,tub-dVP16AD, UAS-2XEYFP; Sp/CyO; Dr/TM3,Sb	Screen Trojan Gal4DBD recombinants on II or III
	yw; tub-dVP16AD, UAS-2XEGFP; Dr/TM3,Sb	Screen Trojan Gal4DBD recombinants on X or III
	yw;tub-Gal4DBD;UAS-2XEGFP	Screen Trojan dVP16AD or p65AD recombinants on X
	yw,UAS-2XEYFP;Sp/Cyo;tub-Gal4DBD	Screen Trojan dVP16AD or p65AD recombinants on II
	yw,UAS-2XEYFP; tub-Gal4DBD; Dr/TM3,Sb	Screen Trojan dVP16AD or p65AD recombinants on III
Lines for use in generating Trojan-MiMIC lines by *in vivo* RMCE	yw,pC-(loxP2-attB2-SA(0)-T2A-Gal4-Hsp70); +; +	Singlet Donor (phase 0) for *in vivo* RMCE with MiMIC inserts on II and III
	yw; pC-(loxP2-attB2-SA(0)-T2A-Gal4-Hsp70); Dr/TM3,Sb	Singlet Donor (phase 0) for *in vivo* RMCE with MiMIC inserts on X and III
	yw; Sp/Cyo; pC-(loxP2-attB2-SA(0)-T2A-Gal4-Hsp70)	Singlet Donor (phase 0) for *in vivo* RMCE with MiMIC inserts on X and II
	yw, pC-(loxP2-attB2-SA(1)-T2A-Gal4-Hsp70); +; +	Singlet Donor (phase 1) for *in vivo* RMCE with MiMIC inserts on II and III
	yw; pC-(loxP2-attB2-SA(1)-T2A-Gal4-	Singlet Donor (phase 1) for *in vivo*

	Hsp70); Dr/TM3,Sb	RMCE with MiMIC inserts on X and III
	yw; Sp/Cyo; pC-(loxP2-attB2-SA(1)-T2A-Gal4-Hsp70)	Singlet Donor (phase 1) for *in vivo* RMCE with MiMIC inserts on X and II
	yw, pC-(loxP2-attB2-SA(2)-T2A-Gal4-Hsp70); +; +	Singlet Donor (phase 2) for *in vivo* RMCE with MiMIC inserts on II and III
	yw; pC-(loxP2-attB2-SA(2)-T2A-Gal4-Hsp70); Dr/TM3,Sb	Singlet Donor (phase 2) for *in vivo* RMCE with MiMIC inserts on X and III
	yw; Sp/Cyo; pC-(loxP2-attB2-SA(2)-T2A-Gal4-Hsp70)	Singlet Donor (phase 2) for *in vivo* RMCE with MiMIC inserts on X and II
	yw, pC-(lox2-attB2-SA-T2A-Gal4-Hsp70)3; +; +	Triplet Donor for *in vivo* RMCE with MiMIC inserts on II and III
	yw; pC-(lox2-attB2-SA-T2A-Gal4-Hsp70)3; Dr/TM3,Sb	Triplet Donor for *in vivo* RMCE with MiMIC inserts on X and II
	yw; Sp/Cyo; pC-(lox2-attB2-SA-T2A-Gal4-Hsp70)3	Triplet Donor for *in vivo* RMCE with MiMIC inserts on X and II
	y, y^{+}-hs-Cre, vas-d ΦC31[1]	Dual recombinase line

[1]The X-linked hs-Cre and vas-dΦC31 transgenes were recombined from individual stocks obtained from the Bloomington Stock Center (#34516 and #40161, respectively). The selection marker, 3XP3-RFP, originally associated with the vas- dΦC31 transgene was flanked by loxP sites and was excised upon recombination with hs-Cre.

Supplemental Experimental Procedures

Molecular Biology

Primers for PCR were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa) and PCR amplification was performed with the 2X PCR Master Mix (Roche) or Phusion (NEB). All restriction enzymes and T4 DNA ligase were from New England Biolabs, Inc and plasmid mini-prep and midi-prep DNA was isolated using the High Pure Plasmid DNA Isolation Kit (Roche) or S.N.A.P. Plasmid DNA Midi Prep Kit (Invitrogen), respectively. All gene synthesis was carried out by Epoch Life Science, Inc (Sugar Land, TX) and DNA sequencing was performed by either the NINDS DNA Sequencing Facility or by Genewiz (Frederick, MD).

Trojan exon Constructs

All Trojan exons except for the pT-GEM construct were made by modifying the three protein trap vectors introduced by Venken et al. for the three different reading frames: pBS-KS-attB1-2-PT-SA-SD-0, pBS-KS-attB1-2-PT-SA-SD-1 and pBS-KS-attB1-2-PT-SA-SD-2 (Drosophila Genomics Resource Center, clones # 1297, 1305 and 1313; Indiana University). To make the three Trojan Gal4 constructs with Hsp70 transcription termination signals (pBS-KS-attB2-SA(0/1/2)-T2A-Gal4-Hsp70), a T2A-Gal4-Hsp70 DNA fragment was PCR amplified from a pC-attB-bursα-mCD8-EGFP-T2A-Gal4 construct (Addgene #39463) (Diao and White, 2012; Venken et al., 2011)using primers with flanking *Bam*H I sites and the gel purified product was digested with *Bam*H I and ligated with each of the CIP-treated *Bam*H I-digested pBS-KS-attB1-2-PT-SA-SD vectors and then transformed into DH5α *E coli*. The Trojan Gal4 constructs lacking the Hsp70 terminator were made identically except that the T2A-Gal4 sequence was amplified without the Hsp70 terminator. Similarly, the Trojan Split Gal4 constructs, Gal4DBD, were made using the same strategy except that the T2A sequence (codon optimized for *Drosophila*

melanogaster) was integrated into the forward primers used to amplify Gal4DBD-Hsp70 and p65AD-Hsp70 fragments from the pBPZpGAL4DBDUw (Addgene #26233) vector(Pfeiffer et al., 2010), respectively. The Trojan dVP16AD constructs were made by first amplifying the dVP16AD sequence from a pCAST-CCAP-dVP16ADZip vector previously used to create pP{y+, dVP16AD} (Gao et al., 2008; Luan et al., 2006b) using a forward primer containing *Bam*H I and dT2A sequences identical to those found in the Gal4 constructs and a reverse primer incorporating sequential *Avr* II, *Pac* I, and *Bgl* II restriction sites. This product was then cut with *Bam*H I and *Bgl* II and subcloned into the *Bam*H I cloning site of each protein trap vector to generate pBS-KS-attB2-SA(0/1/2)-T2A-dVP16AD vectors. Subsequently, the Hsp70 polyA terminator sequence was amplified from pBS-KS-attB2-SA-T2A-Gal4-Hsp70 using forward and reverse primers containing *Pac* I restriction sites and following *Pac* I digestion, this product was subcloned into the *Pac* I site of each pBS-KS-attB2-SA(0/1/2)-T2A-dVP16AD vector to generate pBS-KS-attB2-SA(0/1/2)-T2A-dVP16AD-Hsp70. These vectors are the most versatile of the Trojan vectors made and permit modular replacement of the dVP16AD transgene (using flanking *Apa* I and *Avr* II restriction sites) or the Hsp70 polyA terminator (using *Pac* I).

The Trojan p65AD constructs were made by first PCR amplifying p65AD fragment from pBPp65ADZpUw (Addgene # 26234) vector (Pfeiffer et al., 2010)with primer integrated with T2A sequence and then T2A-p65AD fragment was subcloned into Trojan dVP16AD constructs, which were removed dVP16AD by digestion with *Bam*H I and *Avr* II.

The Trojan QF2 constructs were made by first PCR amplifying QF2 from pattb-nsyb-QF2-Hsp70 (Addgene #46115) (Riabinina, 2015) using in-Fusion (Clontech) compatible oligos (F-T2A-QF2: GAACCCCGGGCCCATGC CACCCAAGCGCAAAAC;
R-T2A-QF2: TTATTAATTAACCTAGGTCACTGTTCGTATGTATTAATGTCGGAGAAG).

The PCR product was in-Fusion cloned into pBS-KS-attB1-2-SA (0/1/2)-T2A-VP16AD-Hsp70 that had been digested with *Nco* I/*Avr* II to remove the VP16AD insert. The Trojan LexA::QFAD constructs were made by first PCR amplifying LexA::QFAD from pattb-nsyb-Lex::QF-Hsp70 (Addgene #46123) (Riabinina, 2015) using in-Fusion (Clontech) compatible oligos (F-T2A-LexQF: GAACCCCGGGCCCATGC CACCCAAGAAGAAGCGAAAAGTAG; R-T2A-QF2: TTATTAATTAACCTAGGTCACTGTTCGTATGTATTAATGTCGGAGAAG). The PCR product was in-Fusion cloned into pBS-KS-attB1-2-SA(0/1/2)-T2A-VP16AD-Hsp70 that had been digested with *Nco* I/*Avr* II to remove the VP16AD insert.

To make the Trojan 3XGal80 constructs, we first synthesized a T2A-Gal80$^{FLAG/HA}$-P2A-Gal80^{EGFP}-T2A-Gal80-Hsp70 sequence with flanking *Bam*H I restriction sites using the P2A and T2A sequences described in Diao and White (Diao and White, 2012) and the drosophilized Gal80 sequence of Pfeiffer et al. (Pfeiffer et al., 2010). This construct was then subcloned into the *Bam*H I site of each of the three protein trap vectors of Venken et al. (Venken et al., 2011) to produce the three pBS-KS-attB2- SA(0/1/2)-T2A-3X Gal80$^{FLAG/HA/EGFP}$-Hsp70 vectors. In each, the first Gal80 sequence is followed by sequences encoding FLAG and HA tags, which result in C-terminal fusions for immuno-detection. The second Gal80 gene is fused to the EGFP sequence.

pT-GEM constructs

To make the pT-GEM vectors, an initial backbone construct (i.e. pBS-KS-attP2-loxP2-3XP3-RFP) was synthesized and cloned into pBS at the *EcoR* V site. This construct included multi-cloning sites at the 5' (*Age* I, *Sph* I, and *Not* I) and 3' (*Asc* I, *Kpn* I, and *Spe* I) ends for insertion of homology arms; nested (and inverted) attP sites; a floxed 3XP3-RFP sequence consisting of three copies of a Pax3 promoter sequence followed by the coding sequence of Red Fluorescent

Protein (Bischof et al., 2007) to serve as a selection marker following transformation, and the portion of the Hsp70 polyA transcription terminator sequence 3' of an endogenous *Nco* I restriction site. The sequence just prior to the *Nco* I site included the 5' attP site and the linker found between the attB site and the splice acceptor in the protein trap constructs of Venken et al.(Venken et al., 2011). This sequence included the *Xba* I site just prior to the splice acceptor site. To complete the three pT-GEM vectors, a SA(0/1/2)-T2A-Gal4-Hsp70 restriction fragment was excised from each of the three pBS-KS-attB2-SA(0/1/2)-T2A-Gal4-Hsp70 vectors by digestion with *Xba* I and *Nco* I and ligated into pBS-KS-attP2-loxP2-3XP3-RFP after double digestion with the same enzymes.

Each of the homology arms of the construct used for pT-GEM insertion into the *pburs* locus consisted of approximately 1 kb of sequence amplified by PCR from genomic DNA of the {nos-Cas9} attP2 line used for embryo microinjection (Ren et al., 2013). The sequences of the PCR primers used for homology arm amplification were:

For-HA_L: AGTCAG ACCGGT TGCGTCGATGATTCTAAAGC

Rev-HA_L: AGTCAG GCGGCCGC TAGAGGCAGGATTCAAACTT

For-HA_R: AGTCAG ACTAGT ATGTTTGCGAGACGAGTTTC

Rev-HA_R: AGTCAG GGCGCGCC TAGAGTTAGCGAACTCTAAG

The guide RNA expression construct (sgRNA) was made by inserting the annealing product of two oligos (ttcgAGAGTTCGCTAACTCTATAG and aaacCTATAGAGTTAGCGAACTCT) into the U6b-sgRNA-short plasmid of (Ren et al., 2013) after digestion with *Bbs* I (New England Biolabs).

Triplet donor constructs, pC-(lox2-attB2-SA-T2A-Gal4-Hsp70)3

The triplet donor construct, was assembled from several synthesized gene fragments. First, a basic backbone consisting of one of each of the three uniquely floxed attB2-SA-Hsp70 sequences was synthesized with the following features: The attB2-SA sequences used to establish the three different reading frames (i.e. phases) of each donor were identical to the corresponding sequences of the protein-trap vectors developed by Venken et al.(Venken et al., 2011). Following attB2-SA sequence, there is a unique triplet of restriction sites: *Age* I/*Sph* I/*Not* I for Phase 0, *Spe* I/*Mlu* I/*Fse* I for Phase 1, and *EcoR* I/*Bsiw* I/*Asc* I for Phase 2. The sequences of the loxP, loxN and lox2272 sites recognized by Cre recombinase were identical to those used by Livet et al.(Livet et al., 2007), including the 20 bp spacer sequences separating neighboring lox sites. The unique 100 bp spacer sequences separating each pair of lox and attB sites were derived from a 600 bp sequence located in the promoter of *nerfin1* and shown to lack any DNA regulatory elements (Kuzin et al., 2009). The backbone construct was cloned into pBS to form pBS-(lox2-attB2-SA-Hsp70)3 with *Xho* I and *Bam*H I restriction sites flanking the insertion and then subcloned into the pCAST vector (Luan et al., 2006b) using *Xho* I and *Bam*H I to generate the pC-(lox2-attB2-SA-Hsp70)3 vector. Also synthesized were genes encoding the three phases of T2A-Gal4 with the following flanking restriction sites: Phase 0 construct: *Age* I and *Sph* I (5'), *Not* I (3'); Phase 1 construct: *Spe* I and *Mlu* I (5'), *Fse* I (3'); Phase 2 construct: *EcoR* I and *Bsiw* I (5'), *Asc* I (3'). Paired combinations of 5' and 3' restriction sites were then used to subclone the three T2A-Gal4 DNA fragments into the complementary sites of the pC-(lox2-attB2-SA-Hsp70)3 vector to make the final donor construct vector, pC-(lox2-attB2-SA-T2A-Gal4-Hsp70)3.

Singlet donor constructs, pC-(loxP2-attB2-SA-T2A-Gal4-Hsp70)

Singlet donor constructs were made similarly to the triplet donor constructs. First, backbone fragments consisting of loxP flanked attB2-SA sequences were synthesized in all three phases using the attB2-SA sequences described by Venken et al. (2011) and the 100 bp spacer sequence separating the loxP and attB sites used in triplet donor. These backbone fragments were first cloned into pBS using *Xho* I and *Bgl* II restriction sites and then subcloned into the pCAST vector (Luan et al., 2006b) using *Xho* I and *Bam*H I to make the pC-(loxP2-attB2-SA) vectors. A T2A-Gal4-Hsp70 fragment derived from the Trojan T2A-Gal4-Hsp70 construct by *Bam*H I digestion was then cloned into the pC-(loxP2-attB2-SA) vectors to make the pC-(loxP2-attB2-SA-T2A-Gal4-Hsp70) singlet donor constructs.

TubP-dVP16AD, TubP-Gal4DBD, and ETH-Gal4 constructs

To permit screening of Trojan Split Gal4 recombinants by fluorescence we made fly lines that carry ubiquitously expressed dVP16AD or Gal4DBD constructs expressed behind the tubulin-1α promoter (TubP). To make these constructs, the pCaSpeR4-tubP-Gal80 vector (gift of Tzumin Lee) was double digested with *Not* I and *Xba* I to excise Gal80, which was replaced by either dVP16ADZip (obtained from pCAST-CCAP-dVP16ADZip by digestion with *Not* I and *Xba* I) or ZipGal4DBDflUw (amplified from pBPZpGal4DBDUw by PCR using primers carrying flanking *Not* I or *Xba* I restriction sites) (Pfeiffer et al., 2010). The resulting pCaSpeR4-TubP-dVP16AD and pCaSpeR4-TubP-Gal4DBD constructs were used to make transgenic fly lines. The ETH-Gal4 construct was made by cloning into the pPTGAL vector (Sharma et al., 2002), a 397 bp promoter fragment starting just upstream of the start methionine codon of the Ecdysis Triggering Hormone gene. This fragment includes a 382 bp sequence known to promote selective expression in the Inka cells (Park et al., 2002) and was amplified from a BAC clone from the RPCI-98 *Drosophila melanogaster* BAC Library

(http://bacpac.chori.org/dromel98.htm) using the following PCR primers: TAGCAGCAGATCAGAAATACTTTGTA (Forward); GCTGATGCGACTGAAGCTTCACCTGA (Reverse) and the High Fidelity Expand Long Template PCR system (Roche).

Fly lines

MiMIC lines were obtained from either the Bloomington Stock Center at Indiana University or the Bellen Lab of Baylor College of Medicine. The TubP-dVP16AD, TubP-Gal4DBD, and ETH-Gal4 lines were made by P-element transformation and insertions of TubP-dVP16AD and TubP-Gal4DBD on chromosomes X, II, and III were isolated and characterized. An ETH-Gal4 insertion on chromosome II was used in the experiments described here. The following lines were described previously: w;+;Burs-Gal4DBD(Luan et al., 2012), and w;Sp/CyO;CCAP-Gal4DBD and yw;elav-Gal4DBD;Dr/TM3,Sb(Luan et al., 2006b). w;+;Rdl^{2-1}-Gal4 and w;Rdl^{2-2}-Gal4/CyO;+ were gifts from Dr. Julie Simpson at JFRC, HMHI. All other stocks, including: yw, M{vas-int.B}ZH-2A; Sb/TM6B, Tb; yw, UAS-2XEYFP; Dr/TM3,Sb; w;+;UAS-2X EGFP, w;10XUAS-IVS-myr::GFP (attP40);+, w; +;10XUAS-IVS-myr::GFP (attP2), w;13XLexAop2-IVS-myr::GFP (VK00005), w;13XLexAop2-IVS-myr-tdTomato (su(Hw)attP5);+, w;+;20xQUAS-mCD8:GFP/TM6B, w;UAS-nlsGFP;+ ; w;+;nucLacZ, w;UAS-CD4-tdTomato were obtained (or derived from stocks) from the Bloomington Drosophila Stock Center at Indiana University. All plasmid injections to generate Trojan-MiMIC lines were performed by Rainbow Transgenic Flies, Inc. or BestGene, Inc. A complete list of transgenic fly lines developed for use in implementing the Trojan system are shown in Table S1.

Lines made by ΦC31-mediated conversion of MiMIC lines by plasmid injection

Midiprep plasmid DNA containing the desired Trojan exon in the correct reading frame was injected either together with ΦC31 plasmid DNA into the embryos of flies bearing MiMIC insertions into genes of interest or into the embryos of flies bearing both the MiMIC insertion and an X-linked transgene expressing ΦC31. Following ΦC31-mediated cassette exchange and isolation of recombinant animals, PCR amplification of the 5' and 3' ends of the insertion site was performed to confirm correct insertion. Amplification at each end was accomplished using a primer complementary to the flanking genomic sequence 1.2-1.5 kb from the MiMIC insertion and an internal primer complementary to either the T2A sequence (T2A-anti: CGCCGCAGGTCAGCAGG) or a region within the Hsp70 PolyA terminator (Hsp70_sense: TTATTCAGTTCCTGGCTTAAGTT). PCR products were evaluated for correct size and in some cases were sequenced to confirm the phases of the inserted Trojan exon. Recombinant transformants were isolated as described in Figure S1, using UAS-2XEYFP, UAS-2XEGFP, 13XLexAop2-myr-tdTomato, and QUAS-mCD8-GFP as the fluorescent expression markers. In some cases, developmental lethality was observed that appeared to derive from high levels of fluorescence protein expression (particularly EYFP) and a second strategy was used to isolate recombinants based on the loss of the y^+ selection marker associated with the MiMIC insertion. In this strategy, G0 flies from plasmid injected embryos were crossed with flies from the yw;Sp/CyO;Dr/TM3,Sb double balancer line and y^- recombinant progeny were isolated. The orientation of the Trojan exon insert was then determined by PCR amplification of the insertion and confirmed by its ability to drive the expression of a fluorescent reporter. This second strategy was also used to screen for MiMIC-T2A-3XGal80$^{FLAG/HA/EGFP}$-Hsp70 recombinants and expression of 3XGal80$^{FLAG/HA/EGFP}$ in these flies was confirmed by immunostaining with anti-

EGFP antibody (Invitrogen). In general, the endogenous fluorescence of the EGFP-tagged Gal80 moiety was too weak to detect in live animals.

Lines made *in vivo* using the triplet donor line

Flies bearing inserts of the triplet donor transgene were made by P-element mediated transformation using pC-(lox2-attB2- SA-T2A-Gal4-Hsp70)3. Lines were established with insertions of the triplet donor on chromosomes X, II, and III. The transgenic line with the 2nd chromosome insert, "Triplet-10," was used as the Trojan Gal4 donor to make the Trojan-MiMIC Gal4 lines described here. The procedure is as described in Figure S1 and uses a dual-recombinase line with X-chromosome inserts of both the Cre recombinase (Siegal and Hartl, 1996) (i.e. Bloomington stock #766) and vas-dΦC31 (Bischof et al., 2007) (Bloomington stock #40161). Due to the high activity of Cre, all crosses can be raised at 25°C.

Pburs^{TGEM}-Gal4 line made using pT-GEM

The Pburs^{TGEM}-Gal4 transgenic flies were made by microinjection into embryos of {nos-Cas9} attP2 flies, which express the Cas9 nuclease in germline cells under the control of the nanos promoter (Ren et al., 2013). Embryos were co-injected with sgRNA and pT-GEM plasmid DNA, and adults were crossed to flies from the yw,UAS-2XEYFP; Sp/CyO; Dr/TM3,Sb reporter-balancer line to isolate transformed larvae that expressed the UAS-2XEYFP reporter and, as adults, the eye-specific marker, Red Fluorescent Protein. These animals were crossed back to flies of the reporter-balancer line to create stable stocks.

Immunostaining, Fluorescence *In Situ* Hybridization, and Confocal Microscopy

Fixation, staining, and confocal imaging of wholemount CNS preparations from third instar larvae or adults were carried out according to previously published procedures (Peabody et al.,

2008). Rabbit anti-vGlut (gift of Dr. Aaron DiAntonio of Washington University in St. Louis) was used at 1:5000 dilution. Guinea pig anti-Eve (Kosman et al., 1998) (gift of John Reinitz) was used 1:500; mouse anti-Pburs (gift of Aaron Hsueh) was used at 1:100, rabbit anti-Bursα and, rabbit anti-CCAP (Luan et al., 2006a) were used at 1:5000 dilution. Mouse anti-LacZ (Promega) and mouse anti-EGFP (Invitrogen) were used at 1:100 dilution. Secondary antibodies labeled with AlexaFluor 488, 568, and 647 (Invitrogen) were used. Fluorescent *in situ* hybridizations were carried out on embryos fixed with 4% paraformaldehyde. Embryos were equilibrated in hybridization buffer (50% RNase free formamide, 1X Denhardt's (RNase free), 500 µg/ml salmon sperm ssDNA (boiled), 250 µg/ml tRNA (boiled), 50 µg/ml heparin, 4X SSC, 0.1% Tween-20, 5% Dextran sulfate in H_2O) for 1 hr at 52^oC before overnight incubation with vChaT riboprobe. The vChaT gene is co-expressed from the same genomic locus as Cha (Kitamoto et al., 1998), and T7 RNA polymerase (New England Biolabs) was used to generate the riboprobe from a 649 bp fragment of vChaT coding sequence amplified by PCR using specific 5' (TTTCTGGAGCCAACCATTTC) and 3' (CAGCAGTATCAGCAGGAGC) primers with a T7 promoter sequence overhang. Hybridized embryos were washed in Washing Buffer (50% RNase free formamide, 2X SSC, 0.1% Tween-20 in H_2O) before blocking (20% Western blocking reagent (Roche) in phosphate buffered saline plus 0.3% Tween-20) for 1 hr at room temperature and incubated overnight with sheep anti-DIG-POD (1:100, Roche) and mouse anti-GFP (1:200, Sigma) at 4^oC. Hybridized riboprobe was then visualized by tyramide amplification (1:50 Tyramide System Amplification System, Perkin Elmer) and anti-GFP using goat anti-mouse AlexaFluor 488 (1:300, Invitrogen). Fluorescent imaging of larval and adult CNS preparations were performed with a Nikon C-1 confocal microscope. Z-series through either the brain or ventral nerve cord of each sample were acquired in 1µm increments using a 20X objective. The

presented images are composites of separately acquired volume-rendered images of the brain and ventral nerve cords. Imaging of late embryonic and early larval ventral nerve cords used a Leica TCS SP5 confocal microscope (x63/ 1.40 N.A. oil immersion objective) and images were acquired with a voxel size of 1.52 x 1.52 x 0.59 μm.

DNA Sequences: Restriction enzyme sites or multiple cloning sites are underlined. Coding sequences are in green; Hsp70 transcription terminator sequences are in red; *lox sequences are italicized*.

Trojan Exon Sequences

attP2-T2A-Gal4-Hsp70-loxP2-3XP3-RFP of pT-GEM (phase 0)

accggtgcatgcgcggccgccccaggtcagaagcggttttcgggagtagtgccccaactggggtaacctttgagttctctcagttgggggc
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ctgacctgggggcgcgccggtaccactagt

Note that the three phases of pT-GEM differ in the short linker sequences between the splice acceptor and T2A sequences. These linker sequences are as follows:
Linker of Phase 0: agGTGGGAGGTTCCGGTGGAAGCGGAGGTAGCGGCGGATCC
Linker of Phase 1: agGTGGCGGAGGTTCCGGTGGAAGCGGAGGTAGCGGCGGATCC
Linker of Phase 2: agGTCGGGAGGTTCCGGTGGAAGCGGAGGTAGCGGCGGATCC

T2A-Gal4-Hsp70 Insertion Sequence
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T2A-Gal4 Insertion Sequence (without Hsp70 transcription terminator)

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T2A-QF2-Hsp70 Insertion Sequence
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T2A-LexA::QFAD-Hsp70 Insertion Sequence
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T2A-Gal4DBD-Hsp70 Insertion Sequence
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T2A-p65AD-Hsp70 Insertion Sequence
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T2A-dVP16AD-Hsp70 Insertion Sequence
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T2A-3X Gal80$^{FLAG/HA/EGFP}$-Hsp70 Insertion Sequence

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gtttggatcc

loxP2-attB2-SA of singlet donor pC-(loxP2-attB2-SA(0))

ctcgag*ataacttcgtataatgtatgctatacgaagttat*caattggcttattgggatttcaatttaagatgctgctaaataaagttagtcacttac
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atttaaatttga*ataacttcgtataatgtatgctatacgaagttat*

Note: the three phases of the pC-(loxP2-attB2-SA) constructs differ only in the short linker sequence between the splice acceptor and *Bam*H I restriction site. The short linker sequences are the same as those used in pT-GEM constructs.

Backbone of Triplet Donor Sequence: (lox2-attB2-SA-Hsp70)3 of pC-(lox2-attB2-SA-Hsp70)3

ctcgag*ataacttcgtataaagtatcctatacgaagttat*caattggcttattgggatttcaatttaagatgctgctaaataaagttagtcactta
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