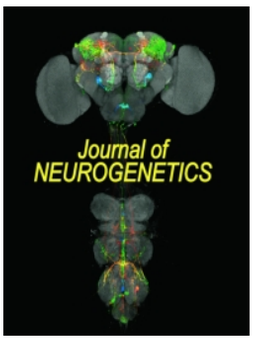

Journal of Neurogenetics

ISSN: 0167-7063 (Print) 1563-5260 (Online) Journal homepage: http://www.tandfonline.com/loi/ineg20

Rationally subdividing the fly nervous system with versatile expression reagents

J. H. Simpson

To cite this article: J. H. Simpson (2016): Rationally subdividing the fly nervous system with versatile expression reagents, Journal of Neurogenetics, DOI: 10.1080/01677063.2016.1248761

To link to this article: http://dx.doi.org/10.1080/01677063.2016.1248761

Published online: 15 Nov 2016.

JOURNAL OF NEUROGENETICS, 2016
http://dx.doi.org/10.1080/01677063.2016.1248761

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Rationally subdividing the fly nervous system with versatile expression reagents

J. H. Simpson[a,b]

[a]Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA, USA; [b]Molecular, Cellular and Developmental Biology Department, University of California, Santa Barbara, Santa Barbara, CA, USA

ABSTRACT

The ability to image and manipulate specific cell populations in Drosophila enables the investigation of how neural circuits develop and coordinate appropriate motor behaviors. Gal4 lines give genetic access to many types of neurons, but the expression patterns of these reagents are often complex. Here, we present the generation and expression patterns of LexA lines based on the vesicular neurotransmitter transporters and Hox transcription factors. Intersections between these LexA lines and existing Gal4 collections provide a strategy for rationally subdividing complex expression patterns based on neurotransmitter or segmental identity.

ARTICLE HISTORY
Received 1 June 2016
Revised 10 October 2016
Accepted 12 October 2016

KEYWORDS
Vesicular neurotransmitter transporters; Hox genes; neural circuit mapping; expression patterns; homologous recombination; P-element replacement; Drosophila

Introduction

The ability to control the expression of transgenes in specific neurons permits powerful interrogation of nervous system anatomy, development and function. *Drosophila* has an array of tools to label and manipulate subsets of neurons, and these reagents are continually being improved. The UAS-GAL4 system (Brand & Perrimon, 1993) allows flexible and reproducible targeting of reporters and effectors to neuron populations, and the initial GAL4 enhancer trap collections produced many different expression patterns (Manseau et al., 1997; Yoshihara & Ito, 2000). Additional transgenic lines were designed using 'cis-regulatory modules' (potential enhancers) of neural genes fused to the GAL4 transcription factor and inserted into the genome randomly with transposons or at defined loci, e.g. with PhiC31 integrase. Some of these GAL4 lines expressed in only a few neurons, while others labeled broader numbers (Pfeiffer, Jenett, et al., 2008). Complex expression patterns can be refined by various intersectional techniques: splitGAL4, LexA, Q, and recombinase-based strategies combine to permit precise targeting of small numbers of neurons (reviewed in Venken, Simpson, & Bellen, 2011).

The ability to subdivide complex Gal4 patterns by functional or spatial criteria would facilitate the rational design of intersectional experiments. Fortunately, some genes have expression patterns that lend themselves to such an application and several approaches have been used to develop reagents capturing these expression patterns. For example, *TH-GAL4* was assembled using a large genomic region around the tyrosine hydroxylase (TH) gene (Friggi-Grelin et al., 2003) and MiMIC transposons inserted into the gene encoding the vesicular monoamine transporter (VMAT) and glutamic acid decarboxylase (GAD1) have been converted so that GAL4 is produced in tandem with the normal gene product separated by a T2A cleavage site (Diao et al., 2015; Diao & White, 2012; Gnerer, Venken, & Dierick, 2015); see Table 1. Here, we report the construction of reagents that attempt to capture the expression patterns of genes that subdivide the nervous system by neurotransmitter identity (the vesicular neurotransmitter transporters (VNT)) or parasegment of origin (Hox transcription factors). Primarily, we made LexA lines by homologous recombination to permit positive and negative intersections with existing GAL4 lines, but other similar tools are also included (Table 1). We describe our methods and show the expression patterns of the resulting transgenic lines. We discuss our validation efforts, as well as their limits, in the hope that both the experiences and reagents may be of use to the community.

Methods

The expression constructs described here were built with several genetic methods as the technology advanced over time.

Enhancer-GAL4 construction

The initial VNT Gal4 lines were assembled by PCR amplification of the ~8 kb regions upstream of predicted starts from genomic DNA preparations of Canton S flies and

CONTACT Julie H. Simpson, Ph.D., Assistant Professor jhsimpson@lifesci.ucsb.edu Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, 3129 Bio II UCEN Rd, Santa Barbara, CA 93106-9625, USA

Table 1. Reagents targeting neurons based on neurotransmitter.

Transgenic line	Description	References
OK371-GAL4 OK371-splitGAL4-AD	Enhancer trap in VGluT locus; SplitGAL4 (VP16-AD) line generated by P-element replacement	Original GAL4 enhancer trap: K.G. Moffat, J.B. Connolly, J. Keane, S.T. Sweeney, and C.J. O'Kane, unpublished data; expression pattern characterization (Mahr & Aberle, 2006). Split (S. Gao, Takemura, et al., 2008)
VGluT-GAL4 VGluT-GAL80	Cloned cis-regulatory element inserted by P-element	(Daniels et al., 2004); alternative P-element versions (Simpson, unpublished) and Vosshall Lab
VGluT-GAL4 VGluT-GAL4-DBD VGluT-GAL80	Converted mimic insertions	(Diao et al., 2015)
VGlut-LexA	Knock-in by ends out homologous recombination	This work
Cha-GAL4 Cha-splitGAL4-DBD Cha-GAL80	Cloned cis-regulatory element inserted by P-element	(Salvaterra & Kitamoto, 2001); splitGAL4 (Luan, Peabody, Vinson, & White, 2006); GAL80 (Kitamoto, 2002)
Cha-GAL4 Cha-GAL4-DBD	Converted mimic insertions	(Diao et al., 2015)
VAChT-LexA	Knock-in by ends out homologous recombination	This work
GAD1-GAL4	Cloned cis-regulatory element inserted by P-element	(Ng et al., 2002)
GAD1-GAL4 GAD1-GAL4-AD	Converted mimic insertions	(Diao et al., 2015)
VGAT-GAL4	Cloned cis-regulatory element inserted by P-element	Simpson, unpublished; used in Fei et al. (2010)
VGAT-LexA	Knock-in by ends out homologous recombination	This work
TH-GAL4	Dopaminergic neurons	(Friggi-Grelin et al., 2003)
TPH-GAL4; TRH-GAL4	Serotonergic neurons	(Alekseyenko, Lee, & Kravitz, 2010; Park et al., 2006)
TbetaH-GAL4	Octopaminergic neurons	(Schneider et al., 2012; Stowers, 2011)
Ddc-GAL4	Dopaminergic and serotonergic neurons	(Li, Chaney, Roberts, Forte, & Hirsh, 2000)
VMAT-GAL4	Cloned cis-regulatory element inserted by P-element	(Simpson, unpublished)
VMAT-GAL4	Converted mimic insertions	(Gnerer et al., 2015)

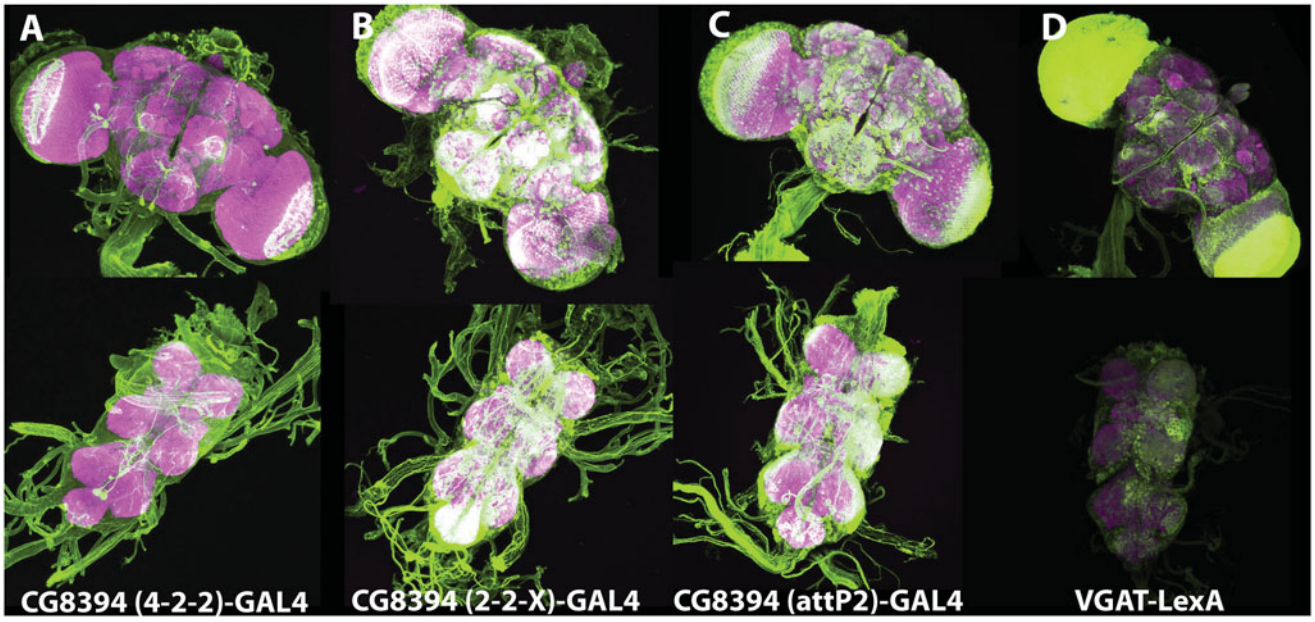

Figure 1. Evolution of VGAT reagents. Expression patterns of two P-element inserts (A and B) and an attP2 insert of CG8394-GAL4 (C) and the VGAT-LexA knock in (D). CG8394 was the name assigned to the gene that was later shown to encode VGAT. The brain and ventral nerve cord of adult flies are labeled with the nc82 neuropil marker in magenta (light grey). The GAL4 and LEXA expression patterns are visualized in green (black) with membrane-targeted UAS-mCD8-GFP and LexAop-myrGFP reporters using an antibody to the green fluorescent protein.

cloned into the pPTGAL vector (Sharma, Cheung, Larsen, & Eberl, 2002) for P-element-based transposon insertion. Approximately five independent inserts were obtained for each construct; expression patterns showed significant position effects (see Figure 1).

5′ENHANCER CG6119 (VMAT)	GGTCTAGACGATGGCCAATGGAGCAGGCTGAGTTG
3′ENHANCER CG6119	CGCGCCGGCGGATTGCAACAAAAAGAATTTGCTCAC
5′ENHANCER CG9887 (VGluT)	GCTCTAGAGGCAACTGGCTGGGCATATTGCCGG
3′ENHANCER CG9887	CGGCGGCCGCCTTGCTGCTCAGCTAGTAGTCCTG
5′ENHANCER CG8394 (VGAT)	TTGCGGCCGCGGAGAGCCACGGCAGATGCCTCTTCG
3′ENHANCER CG8394	GGGGTACCGATGCTGGCTACTAACGGCCCTGATG

For the nSyb-GAL4 and nSyb-GAL80 lines, the putative enhancer region was selected based on (Rao, Lang, Levitan, & Deitcher, 2001).

5' ENHANCER nSyb	CGTCCATCCACTTACATGGCTCTGG
3' ENHANCER nSyb	GGTGTTCGAGTTTAACGCCTGGTAC

P-element replacement

For the P-element replacements, we used a LexA enhancer trap, pPupP65L (constructed by Andrew Seeds), an *X*-linked y + GAL80 #14 (gift of Chris Potter), and *X*-linked

3xP3dsRed-marked splitGAL4s P[JLS-ZpDBD] and P[JLS-ZpAD] (Robinett, Vaughan, Knapp, & Baker, 2010) enhancer traps (constructed by Jon-Michael Knapp). The *tsh*-GAL80, *tsh*-LexA, and *tsh*-splitGAL4-AD lines were made by replacing the Tsh-GAL4 insertion in the 5′ UTR (B#3040) (Calleja, Moreno, Pelaz, & Morata, 1996). For the first replacement (GAL80), one replacement was obtained from >250 candidates. The initial selection was based on second chromosome lethality, which proved misleading because imprecise excision of the initial P-element was common. We then screened by PCR. Interestingly, the y + marker also showed 'harlequin' expression, similar to the w + that marked the original GAL4 insertion. Subsequent P-element replacements were screened by expression pattern when possible.

Homologous recombination

Ends-out homologous recombination (Gong & Golic, 2003) was used to make the LexA knock-in lines. Knock-in donors were assembled by standard cloning procedures. Donors were injected by Rainbow (rainbowgene.com) and mobilized by crossing to transposase. Candidate knock-ins were tested by PCR using primers outside the homology arms and within the inserted sequences so that correct targeting events could be distinguished from the donor insertions. Candidates were also screened by expression patterns and complementation testing with null alleles or deficiencies. Some lines were further characterized by Southern blotting and low-copy number whole genome sequencing.

For the Antp-GAL4 knock in, the donor was located in the Dfd-attP landing site; having the donor and target in close proximity seemed to increase the percentage of successful insertions. For some knock-in lines, the mini-white transgene marker was excised by crossing to hs-B3 recombinase (Nern, Pfeiffer, Svoboda, & Rubin, 2011) and selecting for loss of eye color, with retention of lethality and ability to drive *LexAop-GFP* in the expected expression pattern. The initial LexA knock in construct was replaced by SIRT (G. Gao, McMahon, Chen, & Rong, 2008) and selected based on expression patterns observed when crossing to *UAS-myrGFP, ElaV-SplitGAL4-AD*, or *TubP-splitGAL4-DBD*.

The following deficiencies were obtained from the Bloomington Stock Center (www.flystocks.bio.indiana.edu):

VGAT: Df(2R)BSC401/CyO (B#24425) and Df(2R)Exel7130/CyO (B#7875),
VGluT: Df(2 L)Exel17011/CyO (B#7783)
VAChT: Df(3)Cha M5/TM3Sb (Gailey & Hall, 1989)
Hox Df (removing Dfd, Scr, and Antp) (B#24926)

Most of the stocks used for imaging were generated by B. Pfeiffer and G. M. Rubin (Pfeiffer, Jenett, et al., 2008; Pfeiffer, Ngo, et al., 2010) and are available at the Bloomington Stock Center:

Tub > GAL80–6 > (attP18)
JFRC79: 8xLexAop-FlpL (attP40)
JFRC12: 10xUAS-ivs-myr::GFP (attP2)
JFRC20: 8xLexAop2-ivs-GAL80-wpre (suHWattP5)

Immunohistochemistry

The expression patterns of these lines were assayed by standard adult brain immunohistochemistry and imaging techniques (Hampel, Chung, et al., 2011). *LexA* lines were crossed to *13xLexAop2-nls-LacZ* (Knapp, Chung, & Simpson, 2015) or *LexAop-myr-GFP* (Pfeiffer, Ngo, et al., 2010). Gal4 lines were crossed to *UAS-mCD8-GFP* or *10xUAS-tdTomato-nls*. Primary antibodies: Rabbit anti-GFP (Invitrogen A11122 1:500), Rabbit anti-BetaGal (*Cappel 55976*), *Rabbit anti-tdTomato*, Mouse Bruchpilot (nc82 Iowa Developmental Studies Hybridoma Bank 1:50), and Rat anti-n-Cadherin (DNEX Iowa Developmental Studies Hybridoma Bank). Secondary antibodies (Goat anti-Rabbit 488 A11034 and Goat anti-Mouse 568 A11031) were obtained from Molecular Probes and used at a 1:500 in PBST. Brains, ventral nerve cords (VNC), and larva were dissected in PBS, fixed in 2% paraformaldehyde overnight at 4 °C, washed and blocked in PBT-NGS, incubated overnight with primary antibodies, washed, incubated with secondary antibodies overnight, washed and mounted in Vectashield (Vector Labs, Burlingame, CA). Samples were imaged on a Zeiss Pascal or 700 confocal with a 25× objective and 1 µm sections. The resulting images were processing in ImageJ (NIH, Bethesda, MD) and Adobe Photoshop/Illustrator (Adobe Systems, San Jose, CA) to generate figures.

Results

Subdividing by transmitter

The nervous system of Drosophila uses many of the same neurotransmitters as vertebrates but not always in the same types of neurons (O'Kane, 2011). In Drosophila, motor neurons and some neurons in the central brain release glutamate. Glutamate is packaged into synaptic vesicles by the vesicular glutamate transporter (VGluT) (Daniels et al., 2004); therefore, VGlut is predicted to be expressed in all glutamatergic neurons. In contrast, most sensory and excitatory interneurons use the neurotransmitter acetylcholine. It is synthesized by choline acetyltransferase (ChAT) and loaded into vesicles by the vesicular acetylcholine transporter (VAChT); these genes are encoded by nested transcripts in one of the rare examples of 'operon-like' organization in Drosophila (Kitamoto, Wang, & Salvaterra, 1998). GABA is the primary inhibitory neurotransmitter in Drosophila. It is synthesized by GAD1, and packaged into synaptic vesicles by the vesicular GABA transporter (VGAT) (Fei et al., 2010). Neurons releasing acetylcholine, GABA, and glutamate account for most of the neurons in the brain. To subdivide neuronal populations based on the neurotransmitter they release, we selected the VNT genes as the targets for enhancer fusions and knock-in reagents.

It is challenging to accurately capture the complete spatial and temporal expression pattern of a gene of interest. Other researchers have used large genomic regions (Friggi-Grelin et al., 2003) contained in BACs (Stowers, 2011) or modified transposons that have fortuitously inserted in or near genes of interest (Diao et al., 2015; Gohl et al., 2011). Our initial

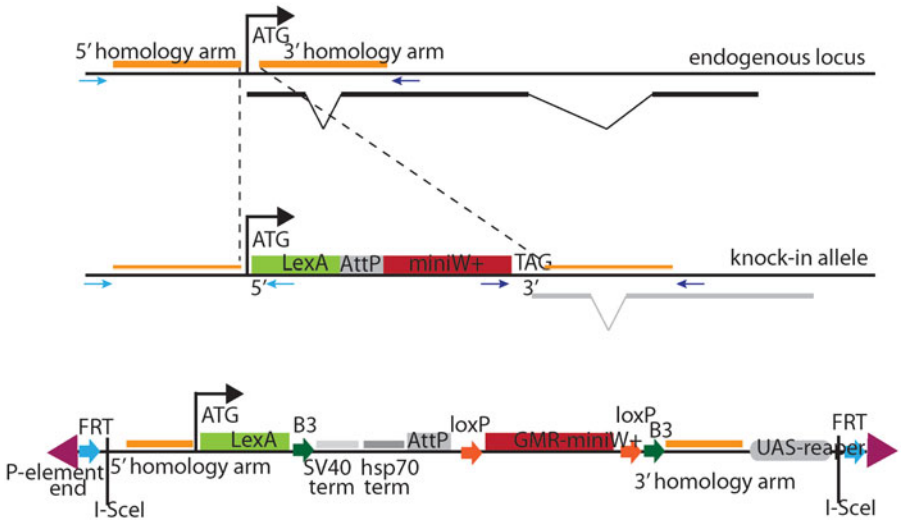

Figure 2. Where to knock-in and what to insert. (A) For a gene of interest, the knock-in cassette is inserted at the start codon of the first protein-coding exon, creating a null allele and capturing the complete expression pattern. Candidates can be tested by PCR using the 5′ and 3′ primer pairs spanning the insert and genomic locus outside of homology arms (blue arrows). (B) The modular donor construct (pGXlexAB3-vntATG) contains the standard FRT recombinase and I-SceI restriction enzyme sites required to excise and linearize the plasmid from the donor insertion as well as numerous options to modify the knock-in locus after transgenic flies have been obtained.

Table 2. Reagents targeting cells based on segment of origin.

Transgenic line	Description	References
Dfd-LexA Dfd-splitGAL4-AD Dfd-splitGAL4-DBD	In adult brain, labels anterior subesophogeal zone. Knock in by ends-out homologous recombination. W + and w − LexA versions. SplitGAL4s generated by SIRT replacement.	This work
Scr-LexA	In adult brain, labels posterior subesophogeal zone and prothoracic legs. Knock in by ends-out homologous recombination. w + and w − LexA versions. AttP site damaged during insertion so no replacement possible.	This work
Antp-P1-GAL4	Cloned cis-regulatory element inserted by P-element	(Andrew, Horner, Petitt, Smolik, & Scott, 1994)
Antp-GAL4	Knock in (minimally characterized)	This work
Ubx-GAL4	P element replacement of LacZ enhancer trap insert	(de Navas et al., 2006)
AbdA-GAL4	P element replacement of LacZ enhancer trap insert	(Hudry, Viala, Graba, & Merabet, 2011)
AbdB-GAL4	P element replacement of LacZ enhancer trap insert	(de Navas et al., 2006)
AbdB-splitGAL4-DBD	P element replacement	This work
Tsh-LexA	Labels thoracic segments, including ventral nerve cord, but not peripheral nervous system or abdominal segments. P-element replacement of GAL4 enhancer trap (Calleja et al., 1996).	GAL80 replacement was made first (Simpson, unpublished) and first used in Clyne and Miesenbock (2008). LexA and splitGAL4-AD: this work.
Tsh-GAL80 Tsh-splitGAL4-AD		
nSyb-GAL4 nSyb-GAL80	Labels post-mitotic neurons. Designed enhancer; various P-element insertions. Similar genomic DNA region to R57C10.	Enhancer initially from Rao et al. (2001). Initial inserts Simpson, unpublished, used in Dietzl et al. (2007).
ElaV-GAL80 ElaV-splitGAL4-AD ElaV-splitGAL4-DBD	'Pan-neuronal'. Designed enhancer; various P-element insertions.	GAL80: Simpson, unpublished. Splits (Luan et al., 2006)

attempts to generate reagents that label neurons based on neurotransmitter used putative enhancer regions (~8 kb of genomic sequence upstream of the predicted transcriptional start sites). These were cloned into the pPTGAL (Sharma et al., 2002) vector and inserted by P-element transposition. As an example, the evolution of VGAT reagents is shown in Figure 1. Multiple insertions were obtained but showed significant position effects on expression patterns (Figure 1(A,B)). The enhancer construct was remade into a phiC31 integrase-compatible vector (Pfeiffer, Jenett, et al., 2008) and inserted into the attP2 landing site, showing an expression pattern similar to the more-broadly expressing P-element insertions (Figure 1(C)). The differences in these expression patterns and uncertainty about how well they capture the actual expression pattern of VGAT lead us to design a knock-in approach (described below); the expression pattern of the VGAT-LexA knock-in is shown in Figure 1(D).

We chose to replace the first coding exon of our target genes by homologous recombination (Gong & Golic, 2003) using a strategy similar to that used to make *Dsx* and *Fru* knock-in alleles (Demir & Dickson, 2005; Manoli et al., 2005); see Figure 2(A). To avoid potential chimeric proteins, which might exhibit neomorphic or dominant negative effects, we replaced the first coding exon with LexA followed by translation and transcription stop signals. Thus, a correctly targeted knock-in is predicted to generate a null allele. As obtaining knock-in events is expected to be infrequent, we opted to insert the LexA transcription factor to facilitate screening for the desired insertion based on the expression pattern of a *lexAop-GFP* reporter. LexA knock-ins have the

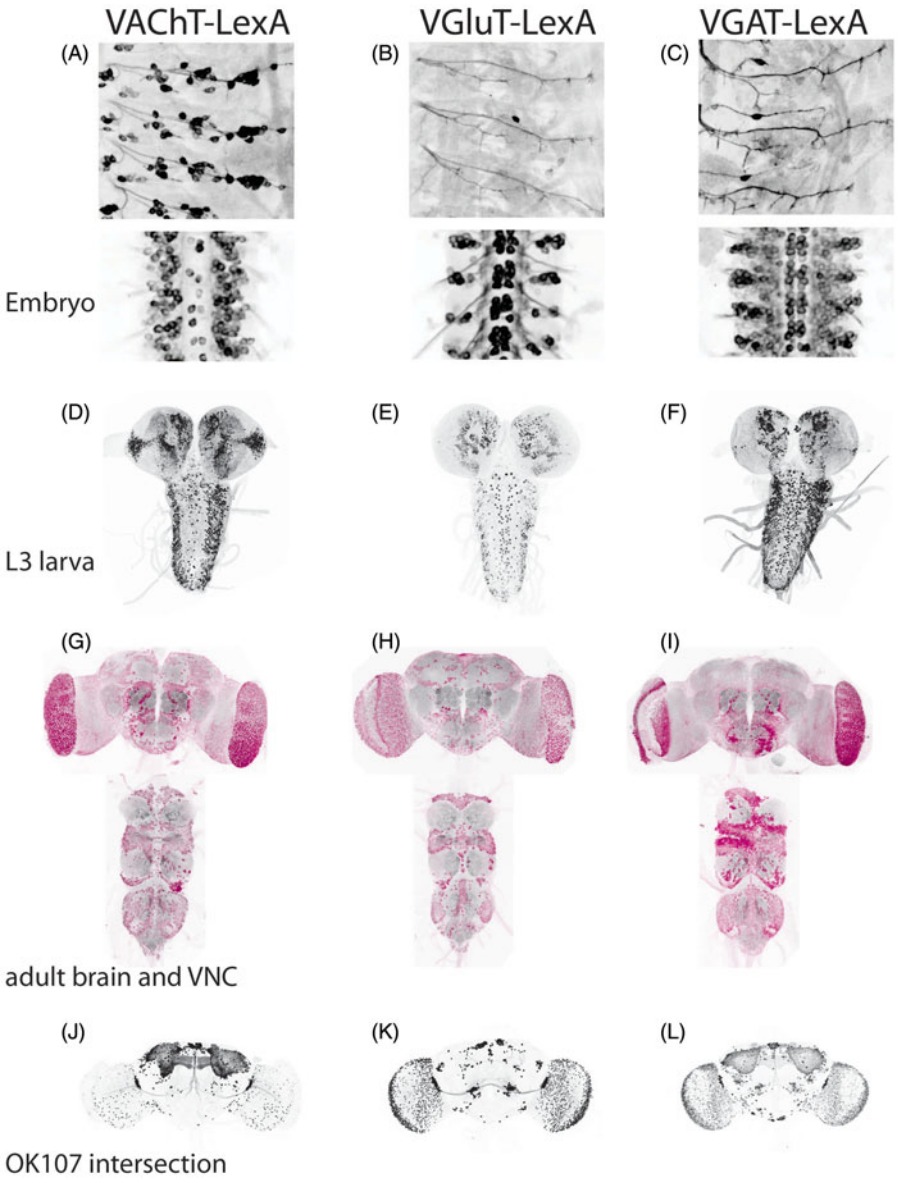

Figure 3. Expression patterns of vesicular neurotransmitter transporter LexA knock-in lines. Embryonic peripheral and central nervous system expression is shown with a membrane-targeted reporter, LexAop-myrGFP (A–C). Larval (D–F) and adult (G–I) expression patterns are visualized with the nuclear reporter LexAop-nls-LacZ (black or magenta). The synaptic neuropil (in grey) was labeled with nc82 (bruchpilot). (J–L) Intersections between the knock-ins and OK107-GAL4 (Connolly et al., 1996) are shown below using 8xLexOp-FlpL (attP40); 10xUAS-FRT-stop-FRT-tdTomato-nls (VK00027).

additional virtue of being useful for performing positive or negative intersectional experiments (Pfeiffer, Ngo, et al., 2010) with existing Gal4 collections.

We included the phiC31 integrase attP sequence in our construct (Figure 2(B)) so that the initial knock-in reagents could be converted to splitGAL4 or GAL80 without undertaking an independent homologous recombination attempt; we performed this replacement successfully to make *Dfd-splitGAL4-AD* and *-DBD* (Table 2). LoxP sites flank the mini-white transgene marker so that it can be removed with cre recombinase once knock-in flies have been isolated, if desired. Likewise, the SV40-α-Tubulin transcription terminators (Stockinger, Kvitsiani, Rotkopf, Tirian, & Dickson, 2005), loxP (or loxP-flanked miniwhite cassette), and attP site can be excised by the B3 recombinase (Nern et al., 2011). This feature was intended to allow use of the target gene's 3′UTR, which may contain post-transcriptional elements that can alter expression of the endogenous gene. In practice, however, we observed that flipping out the B3 cassette led to inconsistent and weak LexA expression, possibly because mRNAs from the knock-in locus were targeted for nonsense-mediated decay (data not shown). Therefore, all knock-in alleles described below included the B3-flanked cassette.

Using ends-out homologous recombination, we obtained LexA knock-in lines of VGluT-LexA, VGAT-LexA, and VAChT-LexA. We confirmed correct targeting by PCR and whole genome sequencing. Complementation testing with deficiencies that remove the target genes showed expected lethality (data not shown).

The expression patterns of the VNT knock-in lines were evaluated in the embryonic, larval, and adult nervous systems using membrane and nuclear GFP reporters, LexAop-myr-GFP (Pfeiffer, Ngo, et al., 2010) and LexAop-nlsLacZ

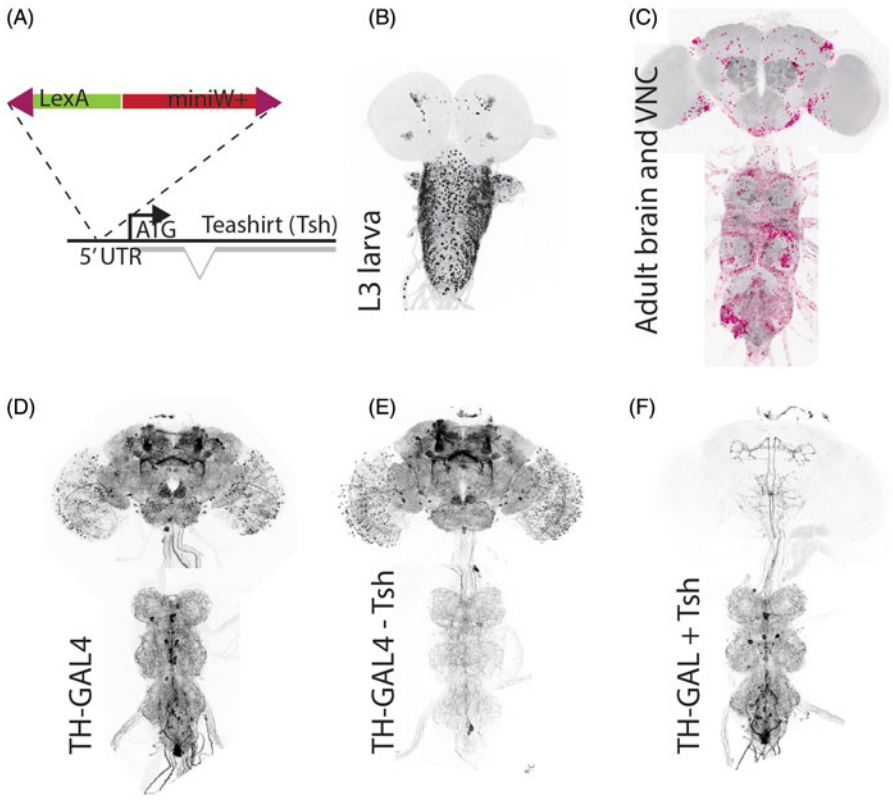

Figure 4. Example use of Tsh-LexA to divide the nervous system. Tsh-LexA is a P-element inserted into the 5′UTR (A). It is expressed in thoracic segments in the larval (B) and adult (C) nervous system. (D) TH-GAL4 (Friggi-Grelin et al., 2003) expresses in dopaminergic neurons throughout the brain and ventral nerve cord. (E) Negative intersection with Tsh-LexA using LexAop-GAL80, UAS-myr-GFP labels descending neurons with cell bodies in the brain, while positive intersection (F) with Tsh-LexA and LexAop-Flp, TubP-FRT-stop-FRT-GAL80, UAS-myr-GFP targets ascending neurons with cell bodies in the ventral nerve cord.

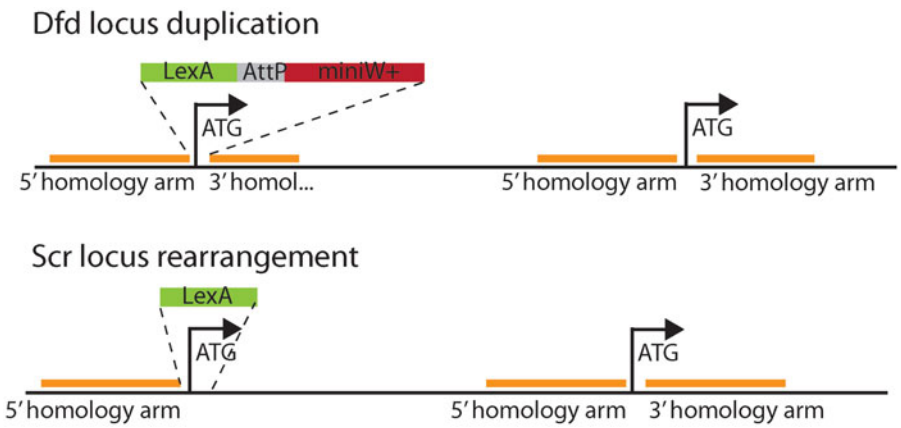

Figure 5. Chromosomal rearrangements of knock-in lines. Whole genome sequencing revealed locus duplication in both the Dfd and Scr knock-ins, and a break within the knock-in cassette in Scr-LexA. The Dfd cassette is 5.5 kb and Scr is 6.4 kb, but the distance between the duplicated regions is not known.

(Knapp et al., 2015). All three lines express in many neurons, but the overall patterns appear different (Figure 3(A–I)). We attempted to assess the fidelity of these reporters by investigating the extent to which neurons are co-labeled by antibodies to GABA, GAD1, VGAT, ChaT, or VGLUT and the corresponding knock-in driver expressing either nuclear or membrane-targeted fluorescent protein reporters (Daniels et al., 2004; Featherstone et al., 2000; Fei et al., 2010; Kolodziejczyk, Sun, Meinertzhagen, & Nassel, 2008). Unfortunately, differences in subcellular localization of the reporters and the broad expression patterns made interpretation challenging: the co-localization was consistent with what is known about transmitters in identified neurons, but was not definitive. For example, VAChT-LexA labels known cholinergic neurons such as the sensory neurons in the embryonic peripheral nervous system, and VGluT-LexA expresses in the known motor neurons (Figure 3(A,B)). While the reagents appear to accurately capture the expression patterns of their target genes, we cannot completely rule out false negatives or false positives when observing the entire expression patterns. The VGAT-LexA remains the most problematic: it shows expression in known GABAergic neurons in the optic lobe, central complex, and olfactory system, but it also shows expression in motor neurons

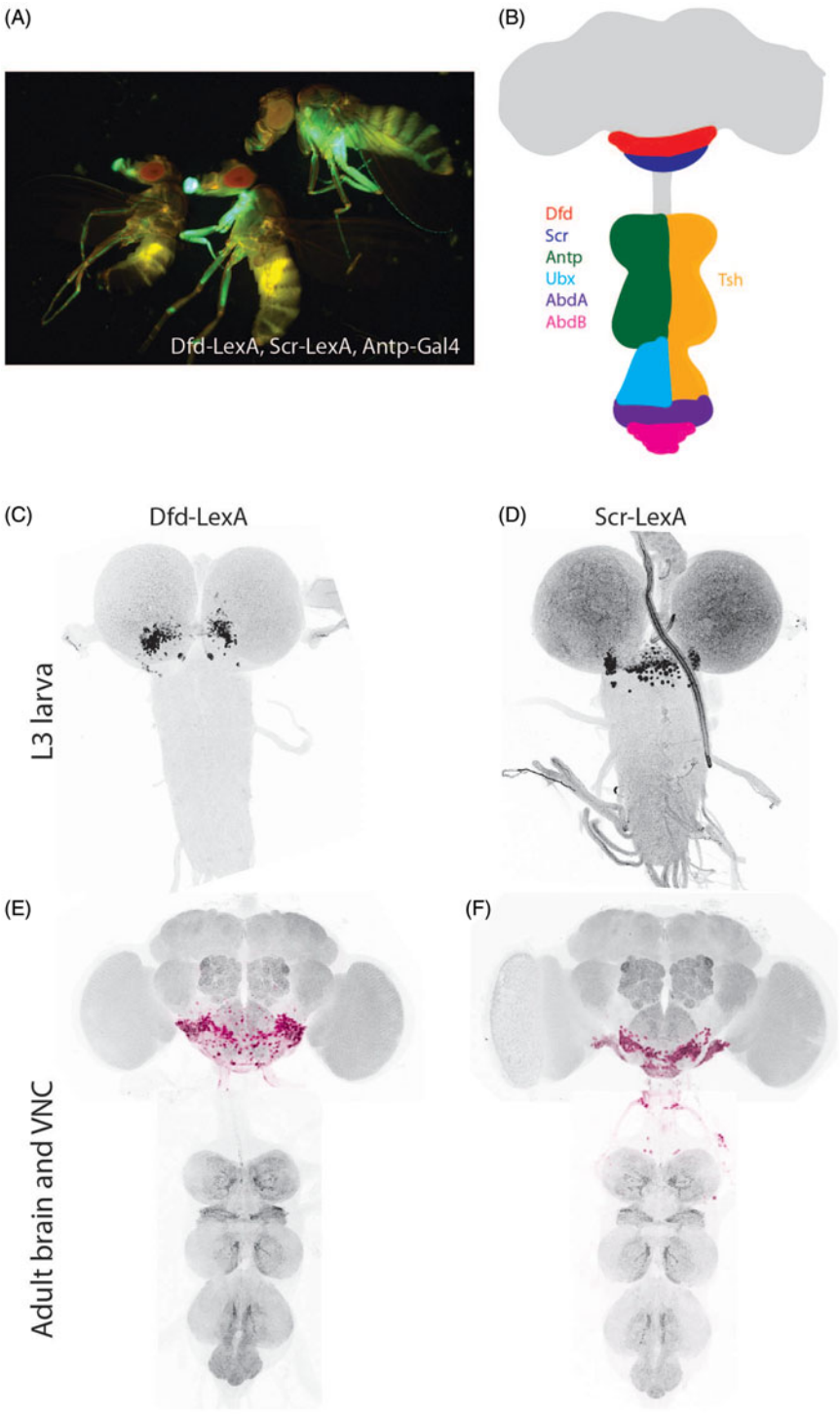

Figure 6. Expression patterns of the Dfd and Scr LexA lines. (A) The expression patterns of Dfd-LexA, Scr-LexA, and Antp-GAL4 in intact adult flies. (B) Cartoon of adult CNS with the expected segmental location of Hox expression (Jarvis et al., 2012) larval (D,E) and adult (F,G) expression patterns of Dfd and Scr knock in lines visualized with a nuclear reporter (black or magenta, with the neuropil nc82 labeled in grey).

(Figure 3(C)), suggesting possible false positives in this line. As broad expression patterns labeling many neurons are difficult to evaluate, we tested the intersection between these lines and the OK107-GAL4 line (Connolly et al., 1996) that expresses in a subset of neurons in the adult brain; the three intersections label different neuronal populations (Figure 3(J–L)) and are consistent with a previous analysis of transmitter identity in these cells (Chou et al., 2010).

Subdividing by parasegment

The adult brain and ventral nerve cord (VNC, also called the thoracico-abdominal ganglion) have a parasegmental origin specified by the homeotic (Hox) transcription factors. We chose the Hox genes as targets in order to take advantage of parasegmental expression to refine complex GAL4 patterns.

To separate the brain and VNC, we selected the Teashirt (Tsh) gene, which is so-named because an enhancer trap

reporter showed expression in trunk segments but not the head or appendages (Fasano et al., 1991). We performed P-element replacement (de Navas, Foronda, Suzanne, & Sanchez-Herrero, 2006; Sepp & Auld, 1999). Tsh-GAL4 and tsh-LexA expression includes some neurons in the brain but many more located within the VNC (Figure 4(A,B)). As an example of how the segmental LexA lines can be used to dissect GAL4 expression patterns, we combined Tsh-LexA with TH-GAL4(Friggi-Grelin et al., 2003) to label ascending and descending dopaminergic neurons (Figure 4(C–E)).

For targeting the subesophageal zone (SEZ), we selected the anterior Hox genes *Deformed* (*Dfd*) and *Sex Combs Reduced* (*Scr*) and performed homologous recombination to insert LexA. We recovered knock-ins to the Hox genes at low frequency. While the *Dfd* donor inserts had near-normal eye color, the knock-ins had light orange eyes, suggesting that chromosomal regulation might suppress expression of the mini-white eye color marker of our transgenes; a similar observation was made by de Navas et al. (2006). We verified correct targeting using PCR extending from the knock-in cassette into the intended locus and confirmed that the knock-in lines were lethal over deficiencies and known mutants. Whole genome sequencing and copy number analyses revealed duplications (Figure 5) that were not apparent from PCR or Southern blotting (data not shown). We visualized the expression patterns with membrane and nuclear localized fluorescent proteins at several developmental stages (Figure 6); Dfd-LexA and Scr-LexA showed expression in different segments in both the larval and adult nervous system, consistent with data from antibody labeling (data not shown).

Discussion

'*Caveat utilitor*': The reagents described here should be considered tools for refining GAL4 lines with complex expression patterns, rather than as verified and faithful labels for particular cell types. They are a complementary alternative to other intersectional genetic strategies based on lineage (Harris, Pfeiffer, Rubin, & Truman, 2015) or perceived anatomical overlap of expression patterns (Hampel, Franconville, Simpson, & Seeds, 2015). We recommend performing the desired intersection between the Gal4 and LexA lines and confirming the accuracy of transmitter identity on the smaller number of neurons labeled, after (Aso et al., 2014; Chou et al., 2010). Our knock-in lines showed chromosomal rearrangements, revealed by whole genome sequencing. We suggest that researchers making knock-ins, whether by homologous recombination or CRISPR (Gratz et al., 2013), investigate the integrity of the resulting locus by sequencing or qPCR-based copy number analysis, since any method that induces double-stranded breaks exposes chromosomes to repair machinery and may produce unexpected rearrangements.

The development of these reagents yielded some interesting biological insights. For example, we saw that the Hox genes show segmentally restricted expression in the adult as well as the embryo. The neurons labeled by the *Dfd-LexA* line appear to project entirely within the SEZ, suggesting that this brain area must receive and send information to the rest of the brain using neurons that originate elsewhere, perhaps marking it as a discrete processing unit. The reagents designed to target GABA inhibitory neurons also express in motor neurons. While there is no evidence that motor neurons release GABA, antibodies to VGAT and GAD1 suggest that these genes may be co-expressed there (Featherstone et al., 2000). We are currently investigating the anatomy and function of neurons labeled by overlapping transmitter reagents.

Our primary research goal is to determine the neural circuits that coordinate sequential motor behaviors, and to do this we need to alter and observe neural activity in small numbers of neurons. Gal4 lines have been critical for these experiments but often target complex populations containing neurons of different types. The intersectional reagents based on split GAL4, recombinases, LexA, and Q have enabled us to identify neurons critical for behavior and show how they are connected into specific neural circuits. The reagents we present here provide additional options for the rational design of intersections based on presumed neurotransmitter subtype or segment of origin.

Acknowledgements

Jon-Michael Knapp, Phuong Chung, Karen Hibbard, and Shingo Yoshikawa made significant and excellent technical contributions to this work. The author thanks Sean Eddy and Elena Rivas for analyzing the results of whole genome sequencing, members of the Janelia FlyCore for assistance with fly wrangling, and Gudrhun Irke and colleagues for immunohistochemistry on some of the knock-in expression patterns. Steffi Hampel made some of the attP insertion constructs, and Andrew Seeds made the LexA enhancer trap starting line. Chris Potter donated the GAL80 P-element enhancer trap line. Khashayar Mozaffari, UCSB, confirmed the complementation testing of the VNT lines. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. Members of the Ganetzky lab, Simpson lab, and many members of the fly community provided feedback during the evolution of this project. Many of the reagents described here have already been widely distributed and we are grateful for feedback about their utility.

Disclosure statement

The authors report no declaration of interest.

Funding

This work was supported by Helen Hay Whitney Post-doctoral fellowship, a L'Oreal Women in Science award, and Howard Hughes Medical Institute funds to JHS as a Janelia Group Leader.

Dedication

My first constructs to target neurons based on the neurotransmitter they produce were designed and build when I was a post-doctoral fellow with Dr. Barry Ganetzky at the University of Wisconsin, Madison between 2001 and 2006. Ling Ling Ho and I injected them to make transgenic flies at the microscope near Barry's fly pushing station. Much of what I know the art of genetics was learned by asking Barry casual questions while we were sitting back-to-back, and much of what I love about doing research science myself and appreciating what the animal and its mutant phenotypes can tell us follows Barry's example.

I also promise to always use the word 'comprise' very carefully. On the occasion of his retirement, I am glad to have the chance to say thanks.

References

Alekseyenko, O.V., Lee, C., & Kravitz, E.A. (2010). Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male *Drosophila melanogaster*. *PLoS One*, *5*, e10806. doi: 10.1371/journal.pone.0010806.

Andrew, D.J., Horner, M.A., Petitt, M.G., Smolik, S.M., & Scott, M.P. (1994). Setting limits on homeotic gene function: Restraint of sex combs reduced activity by teashirt and other homeotic genes. *EMBO Journal*, *13*, 1132–1144.

Aso, Y., Hattori, D., Yu, Y., Johnston, R.M., Iyer, N.A., Ngo, T.T., … Rubin, G.M. (2014). The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife*, *3*, e04577. doi: 10.7554/eLife.04577.

Brand, A.H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, *118*, 401–415.

Calleja, M., Moreno, E., Pelaz, S., & Morata, G. (1996). Visualization of gene expression in living adult Drosophila. *Science*, *274*, 252–255. doi: 10.1126/science.274.5285.252.

Chou, Y.H., Spletter, M.L., Yaksi, E., Leong, J.C., Wilson, R.I., & Luo, L. (2010). Diversity and wiring variability of olfactory local interneurons in the Drosophila antennal lobe. *Nature Neuroscience*, *13*, 439–449. doi: nn.2489[pii]10.1038/nn.248910.1038/nn.2489.

Clyne, J.D., & Miesenbock, G. (2008). Sex-specific control and tuning of the pattern generator for courtship song in Drosophila. *Cell*, *133*, 354–363. doi: 10.1016/j.cell.2008.01.050.

Connolly, J.B., Roberts, I.J., Armstrong, J.D., Kaiser, K., Forte, M., Tully, T., & O'kane, C.J. (1996). Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. *Science*, *274*, 2104–2107. doi: 10.1126/science.274.5295.2104.

Daniels, R.W., Collins, C.A., Gelfand, M.V., Dant, J., Brooks, E.S., Krantz, D.E., & DiAntonio, A. (2004). Increased expression of the Drosophila vesicular glutamate transporter leads to excess glutamate release and a compensatory decrease in quantal content. *Journal of Neuroscience*, *24*, 10466–10474. doi: 10.1523/JNEUROSCI.3001-04.2004.

Demir, E., & Dickson, B.J. (2005). fruitless splicing specifies male courtship behavior in Drosophila. *Cell*, *121*, 785–794. doi: 10.1016/j.cell.2005.04.027.

de Navas, L., Foronda, D., Suzanne, M., & Sanchez-Herrero, E. (2006). A simple and efficient method to identify replacements of P-lacZ by P-Gal4 lines allows obtaining Gal4 insertions in the bithorax complex of Drosophila. *Mechanisms of Development*, *123*, 860–867. doi: 10.1016/j.mod.2006.07.010.

Diao, F., Ironfield, H., Luan, H., Diao, F., Shropshire, W.C., Ewer, J., … White, B.H. (2015). Plug-and-play genetic access to Drosophila cell types using exchangeable exon cassettes. *Cell Reports*, *10*, 1410–1421. doi: 10.1016/j.celrep.2015.01.059.

Diao, F., & White, B.H. (2012). A novel approach for directing transgene expression in Drosophila: T2A-Gal4 in-frame fusion. *Genetics*, *190*, 1139–1144. doi: 10.1534/genetics.111.136291.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., … Dickson, B.J. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature*, *448*, 151–156. doi: 10.1038/nature05954.

Fasano, L., Roder, L., Core, N., Alexandre, E., Vola, C., Jacq, B., & Kerridge, S. (1991). The gene teashirt is required for the development of Drosophila embryonic trunk segments and encodes a protein with widely spaced zinc finger motifs. *Cell*, *64*, 63–79. doi: 10.1016/0092-8674(91)90209-H.

Featherstone, D.E., Rushton, E.M., Hilderbrand-Chae, M., Phillips, A.M., Jackson, F.R., & Broadie, K. (2000). Presynaptic glutamic acid decarboxylase is required for induction of the postsynaptic receptor field at a glutamatergic synapse. *Neuron*, *27*, 71–84. doi: 10.1016/S0896-6273(00)00010-6.

Fei, H., Chow, D.M., Chen, A., Romero-Calderon, R., Ong, W.S., Ackerson, L.C., … Krantz, D.E. (2010). Mutation of the Drosophila vesicular GABA transporter disrupts visual figure detection. *Journal of Experimental Biology*, *213*, 1717–1730. doi: 213/10/1717 [pii]10.1242/jeb.036053

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., & Birman, S. (2003). Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *Journal of Neurobiology*, *54*, 618–627. doi: 10.1002/neu.10185.

Gailey, D.A., & Hall, J.C. (1989). Behavior and cytogenetics of fruitless in *Drosophila melanogaster*: Different courtship defects caused by separate, closely linked lesions. *Genetics*, *121*, 773–785.

Gao, G., McMahon, C., Chen, J., & Rong, Y.S. (2008). A powerful method combining homologous recombination and site-specific recombination for targeted mutagenesis in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 13999–14004. doi: 10.1073/pnas.0805843105.

Gao, S., Takemura, S.Y., Ting, C.Y., Huang, S., Lu, Z., Luan, H., … Lee, C.H. (2008). The neural substrate of spectral preference in Drosophila. *Neuron*, *60*, 328–342. doi: 10.1016/j.neuron.2008.08.010.

Gnerer, J.P., Venken, K.J., & Dierick, H.A. (2015). Gene-specific cell labeling using MiMIC transposons. *Nucleic Acids Research*, *43*, e56. doi: 10.1093/nar/gkv113.

Gohl, D.M., Silies, M.A., Gao, X.J., Bhalerao, S., Luongo, F.J., Lin, C.C., … Clandinin, T.R. (2011). A versatile in vivo system for directed dissection of gene expression patterns. *Nature Methods*, *8*, 231–237. doi: 10.1038/Nmeth.1561.

Gong, W.J., & Golic, K.G. (2003). Ends-out, or replacement, gene targeting in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 2556–2561. doi: 10.1073/pnas.0535280100.

Gratz, S.J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K., Harrison, M.M., … O'connor-Giles, K.M. (2013). Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. *Genetics*, *194*, 1029–1035. doi: 10.1534/genetics.113.152710.

Hampel, S., Chung, P., McKellar, C.E., Hall, D., Looger, L.L., & Simpson, J.H. (2011). Drosophila Brainbow: A recombinase-based fluorescence labeling technique to subdivide neural expression patterns. *Nature Methods*, *8*, 253–259. doi: nmeth.1566[pii]10.1038/nmeth.156610.1038/nmeth.1566.

Hampel, S., Franconville, R., Simpson, J.H., & Seeds, A.M. (2015). A neural command circuit for grooming movement control. *Elife*, *4*, e08758. doi: 10.7554/eLife.08758.

Harris, R.M., Pfeiffer, B.D., Rubin, G.M., & Truman, J.W. (2015). Neuron hemilineages provide the functional ground plan for the Drosophila ventral nervous system. *Elife*, *4*, e04493. doi: 10.7554/eLife.04493.

Hudry, B., Viala, S., Graba, Y., & Merabet, S. (2011). Visualization of protein interactions in living Drosophila embryos by the bimolecular fluorescence complementation assay. *BMC Biology*, *9*, 5. doi: 10.1186/1741-7007-9-5.

Jackson, F.R., Newby, L.M., & Kulkarni, S.J. (1990). Drosophila GABAergic systems: sequence and expression of glutamic acid decarboxylase. *J. Neurochem*, *54*, 1068–1078. doi: 10.1111/j.1471-4159.1990.tb02359.x.

Jarvis, E., Bruce, H.S., & Patel, N.H. (2012). Evolving specialization of the arthropod nervous system. *Proceedings of the National Academy of Sciences of the United States of America*, *109 Suppl 1*, 10634–10639. doi: 10.1073/pnas.1201876109.

Kitamoto, T. (2002). Conditional disruption of synaptic transmission induces male–male courtship behavior in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 13232–13237. doi: 10.1073/pnas.202489099.

Kitamoto, T., Wang, W., & Salvaterra, P.M. (1998). Structure and organization of the Drosophila cholinergic locus. *Journal of Biological Chemistry*, *273*, 2706–2713. doi: 10.1074/jbc.273.5.2706.

Knapp, J.M., Chung, P., & Simpson, J.H. (2015). Generating customized transgene landing sites and multi-transgene arrays in Drosophila using phiC31 integrase. *Genetics*, *199*, 919–934. doi: 10.1534/genetics.114.173187.

Kolodziejczyk, A., Sun, X., Meinertzhagen, I.A., & Nassel, D.R. (2008). Glutamate, GABA and acetylcholine signaling components in the lamina of the Drosophila visual system. *PLoS ONE*, *3*, e2110. doi: 10.1371/journal.pone.0002110.

Li, H., Chaney, S., Roberts, I.J., Forte, M., & Hirsh, J. (2000). Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in *Drosophila melanogaster*. *Current Biology*, *10*, 211–214. doi: 10.1016/S0960-9822(00)00340-7.

Luan, H., Peabody, N.C., Vinson, C.R., & White, B.H. (2006). Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. *Neuron*, *52*, 425–436. doi: 10.1016/j.neuron.2006.08.028.

Mahr, A., & Aberle, H. (2006). The expression pattern of the Drosophila vesicular glutamate transporter: A marker protein for motoneurons and glutamatergic centers in the brain. *Gene Expression Patterns*, *6*, 299–309. doi: 10.1016/j.modgep.2005.07.006.

Manoli, D.S., Foss, M., Villella, A., Taylor, B.J., Hall, J.C., & Baker, B.S. (2005). Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. *Nature*, *436*, 395–400. doi: 10.1038/nature03859.

Manseau, L., Baradaran, A., Brower, D., Budhu, A., Elefant, F., Phan, H., … Selleck, S. (1997). GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of Drosophila. *Developmental Dynamics*, *209*, 310–322. doi: 10.1002/(Sici)1097-0177(199707)209:3<310::Aid-Aja6>3.0.Co;2-L.

Nern, A., Pfeiffer, B.D., Svoboda, K., & Rubin, G.M. (2011). Multiple new site-specific recombinases for use in manipulating animal genomes. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 14198–14203. doi: 10.1073/pnas.1111704108.

Ng, M., Roorda, R.D., Lima, S.Q., Zemelman, B.V., Morcillo, P., & Miesenbock, G. (2002). Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly. *Neuron*, *36*, 463–474. doi: 10.1016/S0896-6273(02)00975-3.

O'Kane, C.J. (2011). Drosophila as a model organism for the study of neuropsychiatric disorders. *Current Topics in Behavioral Neurosciences*, *7*, 37–60. doi: 10.1007/7854_2010_110.

Park, J., Lee, S.B., Lee, S., Kim, Y., Song, S., Kim, S., … Chung, J. (2006). Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature*, *441*, 1157–1161. doi: 10.1038/nature04788.

Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.T., Misra, S., Murphy, C., … Rubin, G.M. (2008). Tools for neuroanatomy and neurogenetics in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 9715–9720. doi: 10.1073/pnas.0803697105.

Pfeiffer, B.D., Ngo, T.T., Hibbard, K.L., Murphy, C., Jenett, A., Truman, J.W., & Rubin, G.M. (2010). Refinement of tools for targeted gene expression in Drosophila. *Genetics*, 735–755. doi: 10.1534/genetics.110.119917.

Rao, S., Lang, C., Levitan, E.S., & Deitcher, D.L. (2001). Visualization of neuropeptide expression, transport, and exocytosis in *Drosophila melanogaster*. *Journal of Neurobiology*, *49*, 159–172. doi: 10.1002/neu.1072.

Robinett, C.C., Vaughan, A.G., Knapp, J.M., & Baker, B.S. (2010). Sex and the single cell. II. There is a time and place for sex. *PLoS Biology*, *8*, e1000365. doi: 10.1371/journal.pbio.1000365.

Salvaterra, P.M., & Kitamoto, T. (2001). Drosophila cholinergic neurons and processes visualized with Gal4/UAS-GFP. *Brain Research: Gene Expression Patterns*, *1*, 73–82. doi: 10.1016/S1567-133X(01)00011-4.

Schneider, A., Ruppert, M., Hendrich, O., Giang, T., Ogueta, M., Hampel, S., … Scholz, H. (2012). Neuronal basis of innate olfactory attraction to ethanol in Drosophila. *PLoS One*, *7*, e52007. doi: 10.1371/journal.pone.0052007.

Sepp, K.J., & Auld, V.J. (1999). Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in *Drosophila melanogaster*. *Genetics*, *151*, 1093–1101.

Sharma, Y., Cheung, U., Larsen, E.W., & Eberl, D.F. (2002). PPTGAL, a convenient Gal4 P-element vector for testing expression of enhancer fragments in drosophila. *Genesis*, *34*, 115–118. doi: 10.1002/gene.10127.

Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirian, L., & Dickson, B.J. (2005). Neural circuitry that governs Drosophila male courtship behavior. *Cell*, *121*, 795–807. doi: 10.1016/j.cell.2005.04.026.

Stowers, R.S. (2011). An efficient method for recombineering GAL4 and QF drivers. *Fly (Austin)*, *5*, 371–378. doi: 10.4161/fly.5.4.17560.

Venken, K.J., Simpson, J.H., & Bellen, H.J. (2011). Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron*, *72*, 202–230. doi: 10.1016/j.neuron.2011.09.021.

Yoshihara, M., & Ito, K. (2000). Improved GAL4 screening kit for large-scale generation of enhancer-trap strains. *Drosophila Information Service*, *83*, 199–202.