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A GAL4 Driver Resource for Developmental and Behavioral Studies on the Larval CNS of *Drosophila*

Hsing-Hsi Li,[1] Jason R. Kroll,[1,2] Sara M. Lennox,[1,3] Omotara Ogundeyi,[1] Jennifer Jeter,[1] Gina Depasquale,[1] and James W. Truman[1,*]
[1]Janelia Farm Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147, USA
[2]Present address: Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA
[3]Present address: Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA
*Correspondence: trumanj@janelia.hhmi.org
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SUMMARY

We report the larval CNS expression patterns for 6,650 GAL4 lines based on *cis*-regulatory regions (CRMs) from the *Drosophila* genome. Adult CNS expression patterns were previously reported for this collection, thereby providing a unique resource for determining the origins of adult cells. An illustrative example reveals the origin of the astrocyte-like glia of the ventral CNS. Besides larval neurons and glia, the larval CNS contains scattered lineages of immature, adult-specific neurons. Comparison of lineage expression within this large collection of CRMs provides insight into the codes used for designating neuronal types. The CRMs encode both dense and sparse patterns of lineage expression. There is little correlation between brain and thoracic lineages in patterns of sparse expression, but expression in the two regions is highly correlated in the dense mode. The optic lobes, by comparison, appear to use a different set of genetic instructions in their development.

INTRODUCTION

Many of the experimental approaches used in genetic model organisms such as mice and *Drosophila* to study the nervous system benefit from the expression of reporter constructs, or tools to manipulate function, in defined sets of cells. The pioneering method for achieving this goal in *Drosophila* combined the GAL4-UAS expression system (Brand and Perrimon, 1993) and the transposon-mediated enhancer trap method (O'Kane and Gehring, 1987) to generate collections of lines in which the expression pattern of GAL4 was determined by the endogenous regulatory sequences at the genomic insertion site in each line (reviewed in Ito et al., 2003). Expression tools became more refined with the development of the phiC31 integrase system (Groth et al., 2004; Pfeiffer et al., 2008) that allowed the repeated insertion of DNA constructs in the same site in the genome. Use of a single landing site for constructs allows comparison of expression patterns of different *cis*-regulatory regions with minimal concern for "position effects" and with high confidence that different effecter genes (for example, LexA instead of GAL4) driven by the same *cis*-regulatory module (CRM) and inserted at the same site would have the same expression pattern (Pfeiffer et al., 2010). This modularity is critical for employing intersectional strategies to subdivide neuronal populations (see for example, Luan et al., 2006; Pfeiffer et al., 2010) to generate the sparser density of expressing cells needed to identify neurons whose activity is important for a specific behavior.

A large collection of lines, representing about 7,000 CRMs, has been generated in this manner in the Rubin laboratory (Pfeiffer et al., 2008; Jenett et al., 2012) and is available from the *Drosophila* stock center in Bloomington, Indiana (http://flystocks.bio.indiana.edu/Browse/misc-browse/Janelia.php). Moreover, the expression patterns generated by these lines in the adult brain and ventral nervous system (VNS) (Jenett et al., 2012), the embryonic nervous system (Manning et al., 2012), and larval imaginal discs (Jory et al., 2012) are published and available at http://www.janelia.org/gal4-gen1.

In *Drosophila*, the larval and adult nervous systems are adapted for controlling the behavior of two radically different animals. The larva has a reduced sensory system, relying primarily on mechanoreception, and crawls by means of peristaltic waves along its abdomen. The adult, by contrast, has an elaborate sensory system that is dominated by the visual system and adult locomotor functions have been assumed by the thorax with its legs and wings. Accordingly, compared to the ~150,000 neurons in the adult CNS, the larval CNS contains about 12,000 neurons. The larval visual centers are rudimentary, but the olfactory and gustatory centers are well developed. The VNS (also called the ventral nerve cord), especially the abdominal neuromeres, accounts for about 80% of the central neurons of the larva. At metamorphosis, the majority of abdominal neurons die, and this region becomes greatly reduced as the adult abdomen loses locomotor function. By contrast, about 4,500 neurons are added to each thoracic neuromere to accommodate the enhanced sensory and motor demands of walking and flight (Truman et al., 1993).

The new neurons in the adult central brain and VNS are generated by a set of persisting embryonic neuroblasts (NBs). There is a fixed number of NBs in the larval CNS, and each has a unique

identity (Truman et al., 2004; Pereanu and Hartenstein, 2006; Kuert et al., 2014). Each undergoes a short, initial set of divisions during embryogenesis to make a set of larval neurons and then an extended proliferation period during larval growth and early metamorphosis for making adult-specific neurons (Prokop and Technau, 1991). Most of the NBs are type I NBs that have a simple pattern of division; each division of the NB produces a precursor cell, the ganglion mother cell (GMC), that divides once to produce two daughters, A and B, which differ based on Notch signaling (Spana and Doe, 1996; Truman et al., 2010). For lineages in both the brain and VNS, the A and B daughters are typically very different, but within the A and the B groups the cells are much more homogeneous and are referred to as the A and B hemilineages. The neurons that are born during the postembryonic phase extend their axons to an initial target and then arrest. Therefore, late in larval life one finds age-stratified clusters of postembryonic neurons with an apical crown of the NB and GMCs and a basal "tail" of one or two axon bundles inserted into the neuropil. At metamorphosis, the adult CNS is constructed from these clusters of immature neurons and from a subset of remodeled larval neurons (Truman et al., 1993).

Despite its reduced numbers of neurons, the larval CNS has most of the neuronal classes found in the adult. In the larva, though, the number of neurons in a given class is typically reduced to one or two cells, rather than the many neurons per class found in the adult. For example, the larval olfactory system has only a small fraction of the number of neurons found in the adult system, but its level of cellular complexity is similar (Gerber and Stocker, 2007). Thus, the *Drosophila* larva presents a simplified but realistic ground plan for brain and VNS organization.

This paper reports the larval CNS expression patterns of a collection of 6,650 GAL4 lines. Because this GAL4 collection provides expression patterns significantly sparser than conventional enhancer trap lines, and the larva contains only a small fraction of the neurons found in the adult, many larval lines have sparse enough expression to yield meaningful behavioral data when used in their present form. With the data reported in this publication, we are now able to compare the expression patterns driven by thousands of CRMs in the CNS at both larval and adult stages providing insights into the origins of particular adult cell types, as we illustrate for the astrocyte-like glia of the VNS.

Expression in the larval CNS includes not only mature glia and the neurons that mediate larval behavior, but also the clusters of immature neurons and glia that are stockpiled for future use in the adult. Comparison of the lineage expression patterns within the collection of CRMs allowed us to assess how the genetic instructions for making different types of neurons might overlap. This analysis revealed that there is both a dense and a sparse code of information for making the major regions of the nervous system. Brain and thoracic lineages differ in their sparse code but appear to use the same dense code. The optic lobes, by comparison, appear to use a very divergent set of genetic instructions.

RESULTS AND DISCUSSION

Typically, four to five nervous systems were stained for each GAL4 line, and two to three were imaged using confocal microscopy. For each line that showed expression, we selected a representative example and included a Z-projection of its GAL4 pattern, a rotational movie of the projection from −30° to +30°, and a movie of a translation through the stack with the GFP expression and two reference markers, DN-cadherin to show the neuropil (Iwai et al., 1997) and neurotactin (Hortsch et al., 1990b) or neuroglian (Hortsch et al., 1990a) to stain bundles of axons and immature neurites. These images and movies are available at http://www.janelia.org/gal4-gen1 (Figure S1). The larvae were of mixed sex. The only sex-specific dimorphisms that we noted in the larval CNS were in the terminal abdominal lineages that make neurons to control the adult genitalia (Taylor and Truman, 1992).

It is important to stress that although each CRM in the collection is associated with a particular gene, it is unlikely that any CRM placed out of context in an attP site faithfully recapitulates the entire expression pattern of the parent gene (see Pfeiffer et al., 2008). For example, the *gooseberry* gene shows embryonic expression in the NB lineages of rows 5 and 6 (Gutjahr et al., 1993), and in the larva we find strong gooseberry immunoreactivity in postembryonic lineages 3, 5, 6, 12, 19, 20, and 22. The R95D07 line contains an enhancer fragment from the *gooseberry* gene (Figure 1D), and it drives expression in all of these lineages, but also in hemilineage 8A, a hemilineage not included in the gooseberry immunostaining. Therefore, the collection provides a reliable resource for directing expression within the larval CNS, but the use to specific lines to identify patterns of gene expression is problematical.

Patterns of Expression in Larval Neurons

Over 90% of the 6500 lines in the collection showed CNS expression, and Figure 1 shows examples of selected expression patterns. Cellular expression for larval neurons ranged from extremely sparse expression in one or two cells in the brain or VNS (Figures 1A and 1B), to very dense expression. The intensity of expression ranged from weak and stochastic to robust and stereotyped. We scored separately the expression in the brain (the supraesophageal complex including the proto-, deutero-, and tritocerebral ganglia), and the four VNS regions defined by the subesophageal, thoracic, abdominal, and terminal neuromeres. This terminology differs from the adult in which the combined supra- and subesophageal ganglia are often referred to as the "brain." We considered a region as being of interest if it showed medium to strong expression in less than 1% of the neurons in that region. For the brain, this typically meant about ten neurons or less per hemisphere and for the VNS, three neurons or less per hemisegment. We also included lines with more than 1% of the neurons in a region if the neurons were in a similar class or if the rest of the CNS had little or no expression. In scoring the sparseness of neuronal expression, we ignored the clusters of arrested immature neurons because they do not contribute to larval behavior. The distribution of these adult-specific lineages (Figures 1C and 1D) was scored separately. We also noted lines that expressed in the neurons of the optic lobes, mushroom bodies (MBs; Figures 1E and 1F), and central complex. The number of lines that were of interest for the brain, subesophageal, thoracic, abdominal, and terminal regions were 773, 573, 728, 904, and 228 lines, respectively. Expression in

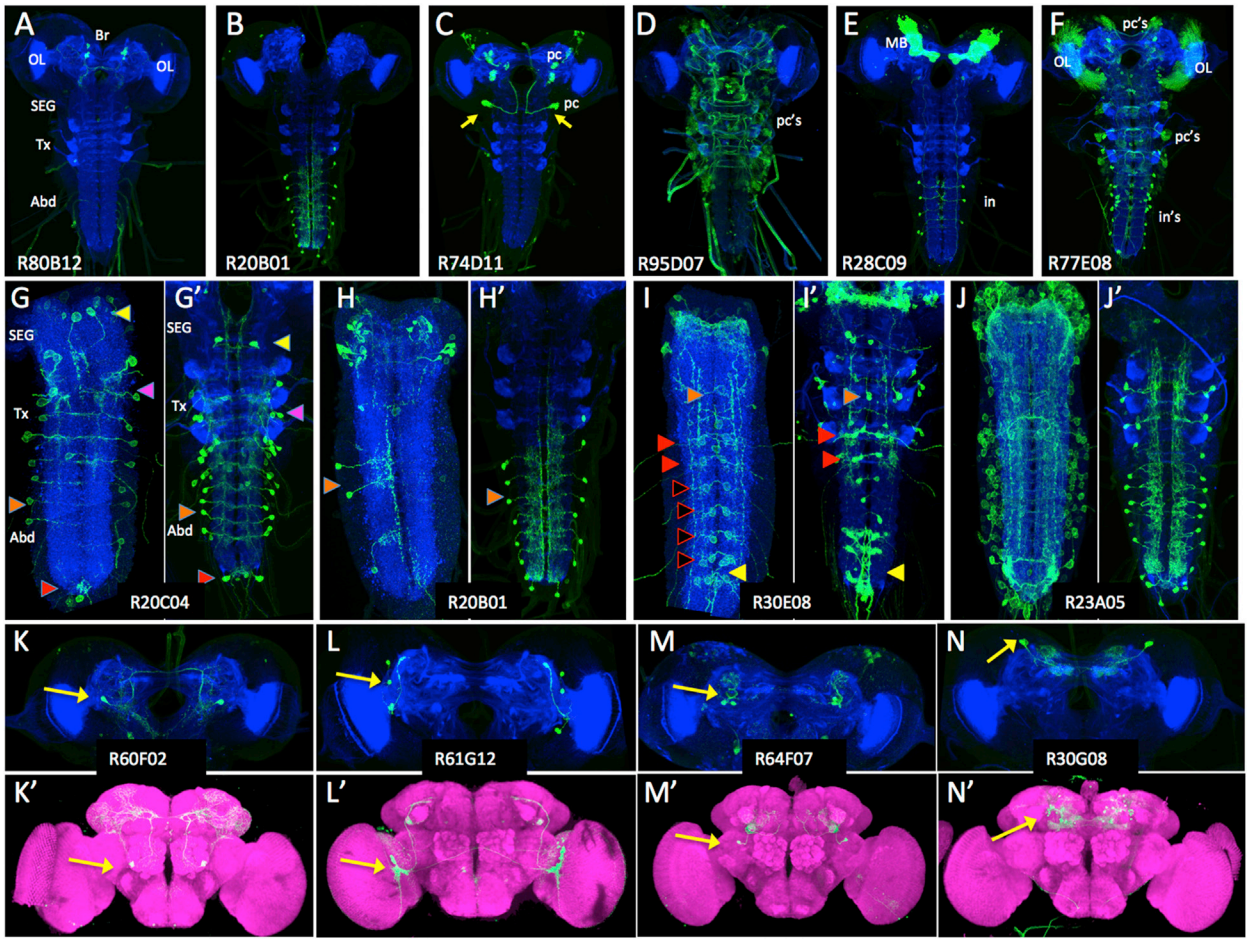

Figure 1. Z-Projections of Confocal Stacks of Larval Nervous Systems Illustrating Selected Expression Patterns in the GAL4 Collection and Examples of Stage-Related Variation and Stability of Expression

(A and B) Extremely sparse lines with expression in a single neuron class in (A) the brain or (B) the abdominal neuromeres.

(C and D) Lines with expression in clusters of postembryonic neurons (pc). Arrows show the subesophageal LB5.

(E) Strong expression in mushroom bodies (MB) as well as abdominal interneurons (in).

(F) Expression in the optic lobes (OL), as well as postembryonic lineages and larval interneurons.

(G–J) Examples from the ventral CNS showing different ways in which expression changes from the beginning (X) to the end (X′) of larval life. For a given GAL4 line, triangles of the same color identify neurons of the same type. (G) All of the neurons seen in the third instar are already expressing at hatching. (H) Expression in a class of interneurons increases during larval growth. (I) At hatching, motoneuron pairs are expressing from segments t3 through a6, but by the last larval stage only the anterior cells are expressing (filled red triangles). (J) A large number of interneurons are expressing at hatching, but only a single pair per segment is expressing in the late larva. Br, brain; SEG, subesophageal ganglia; Tx, thoracic ganglia; Abd, abdominal ganglia.

(K–N) Brain examples showing persistence of expression through metamorphosis from larva (X) to the adult (X′). Arrows indicate the same neuron or neuronal class. (K) Giant deuterocerebral serotonin interneuron. (L) The small vLN circadian clock cells. (M) The larva has two pairs of extrinsic MB neurons with projections in the peduncle and calyx, whereas only one pair expresses in the adult. (N) In the larva, expression is seen in two pairs MB extrinsic neurons, whereas the adult has a small cluster of similar cells.

Green, GFP; blue, N-cadherin; magenta, NC82. The adult images are from Jenett et al. (2012) (http://www.janelia.org/gal4-gen1). See Figure S1 for a screen shot of the larval website and Table S1 for the number of lines for each region of interest.

the CNS regions was not tightly correlated so a line that had sparse expression in the brain might have dense expression in the abdomen and vice versa (see Table S1).

Larval life lasts about 4 days, during which time the larva increases 1,000-fold in weight. The larval neurons increase in size and complexity (Zwart et al., 2013) through this period and clusters of adult-specific neurons begin forming early in the second larval stage (Truman and Bate, 1988). Our larval images are typically from wandering, third-stage larvae just prior to the start of metamorphosis. To determine how consistent expression was through larval growth, we selected 370 lines and compared neuronal expression in the newly hatched first instar larva with that of the wandering third instar (Figures 1G–1J). Focusing primarily on expression in the VNS, we found that 14% of the lines showed no correspondence between the neurons seen at hatching versus at the end of larval life. The remaining lines showed at

least some neurons that expressed consistently through larval growth. Thirty-eight percent of the total showed a pattern of neuronal expression that was essentially the same at the two times (Figure 1G). In 15% of the lines, some neurons of the mature pattern were evident at hatching, but most appeared later during larval growth (Figure 1H). The reverse situation was seen for 33% of the lines in that the hatchling pattern contained all of the L3 neurons but also additional cells that disappeared as the larvae grew. For example, newly hatched larvae of line R30E08 showed expression in a pair of motoneurons present in segments from T3 through A6, but by wandering only the T3 and A1 cells were still expressing (Figure 1I). In other lines, the extra cells that are present at hatching have no apparent relationship to the cells that are sustained through larval life (Figure 1J). These cells likely represent an embryonic expression pattern that then fades in the larva.

When examined in the adult, only 12% of the above lines showed neural elements that we could track back to hatching. This small percentage likely reflects the fact that enhancers control temporal as well as spatial aspects of gene expression (e.g., Figure 3 of Pfeiffer et al., 2008) but also the extensive loss of VNS neurons that occurs at metamorphosis. We tried to reduce the impact of cell death by focusing on the brain, which shows less cell death than does the VNS. Using the Jenett et al. (2012) images for the adult, we compared the larval and adult brain expression for an additional 163 lines that had sparse expression in the larval brain. In 28% of the lines, we could identify neurons or groups of neurons in the adult brain that corresponded to larval cells (Figures 1K–1N). In R61G12, for example, the four small vLN clock neurons (Helfrich-Förster and Homberg, 1993) clearly persist through metamorphosis and are seen in the adult pattern (Figure 1L). Also, the giant deuterocerebral serotonin cell, (Roy et al., 2007) revealed in line R60F02 continues to express in the brain of the adult (Figure 1K). In R64F07, two pairs of cells project to the peduncle and then to the calyx of the mushroom bodies (MBs): this is reduced to a single pair of cells in the adult (Figure 1M). A contrary example is R30G08, which drives expression in two pairs of input neurons that project from the dorsomedial protocerebrum to the medial lobe of the MB. In the adult, expression of R30G08 is seen in a large cluster of cells having a similar projection pattern (Figure 1N). In this last case, we do not know if the larval cells die and are replaced by the adult cluster, or if the larval cells are remodeled and added to the additional adult-specific cells. Nevertheless, the existence of a large collection of larval and adult images based on the same CRMs provides a valuable resource for identifying candidate lines that allow cells to be tracked through metamorphosis.

The Mushroom Bodies and Central Complex

The larva hatches with approximately 300 cells in each MB (Technau and Heisenberg, 1982). Proliferation occurs throughout larval life and metamorphosis (Truman and Bate, 1988; Ito and Hotta, 1992), resulting in about 2,500 neurons in the adult structure. There are four neuroblasts that contribute neurons to each MB, and processes from new neurons occupy the central-most regions of the peduncle and vertical and medial lobes (Kurusu et al., 2002). Hence, the peduncle has a temporally stratified structure with the oldest axons on the periphery and the youngest ones in the central core. The collection contains 584 lines that show expression in the neurons of the mushroom bodies, and Figure 2 shows examples of mushroom body lines that express in various temporal classes. R33E08 and R14F09 typify lines in which expression is only seen in the earliest-born cells that are located at the periphery of the peduncle and whose axons outline the medial and vertical lobes (Figure 2A and 2B). By contrast, R12D02 shows the complementary expression pattern, being absent from the oldest neurons but expressing strongly in all of the younger cells including the NBs and their GMCs (Figure 2C). More restricted populations of young cells are shown in Figures 3D–3F in examples that do (Figure 2D) or do not (Figures 2E and 2F) include the NB, GMCs, and newborn neurons, whose initial neurites occupy the central core of the peduncle.

Larvae lack the tripartite central complex of the adult; however, the precursors of that structure can be identified in the larva (Young and Armstrong, 2010). Lines such as R09D11 express in the type II neuroblasts that produce the neurons for the central complex as well as other structures. Also, other lines that we scored as central complex lines contain a subset of neurons from these lineages that are devoted exclusively to central complex. Their bundled axons extend across the midline (Figure 2G), and their processes initially partition out into eight domains, four on either side of the midline.

The Visual System and Optic Lobes

The adult visual system is composed of the photoreceptors of the compound eyes and the four optic lobe neuropils: the lamina, medulla, lobula, and lobula plate. By late in the last larval stage, the larval photoreceptors have been joined by ingrowing photoreceptors from the differentiating eye imaginal disc (Figures 2H and 2I). Depending on their position in the ommatidium unit, the photoreceptors project either to the lamina (R1 to R6) or to the medulla (R7 and R8). Some lines detect all of the photoreceptors (Figure 2H), whereas others are selective for particular photoreceptor types, such as R83H05 that expresses only in R7 and R8 (Figure 2I). Unlike the NBs for the rest of the CNS, the optic lobe NBs come from an epithelial placode that invaginates during embryogenesis (Green et al., 1993). This placode forms discrete inner and outer proliferation zones, with the former making the neurons of the lamina and outer medulla and the latter generating neurons for the inner medulla, lobula, and lobula plate (Minertzhagen and Hansen, 1993). The collection contains lines that express in both proliferation zones (Figure 2L), or just the outer (Figure 2M) or the inner proliferation zone (Figure 2P). In the latter cases, it may be with or without their associated progeny. Also shown in Figure 2 are different collections of postmitotic neurons from the lamina (Figures 2J and 2K), the medulla (Figures 2N and 2O), and the lobula and lobula plate (Figure 2Q). The neurons in lines depicted in Figures 2O and 2Q carry output from the optic lobe to medial brain structures.

Glial Expression

The GAL4 collection has 268 lines that express in diverse classes of glia (terminology from Klämbt, 2009). These include the perineurial and subperineurial glia of the outer sheath, the ensheathing glia that wrap central axon bundles, the astrocyte-like glia

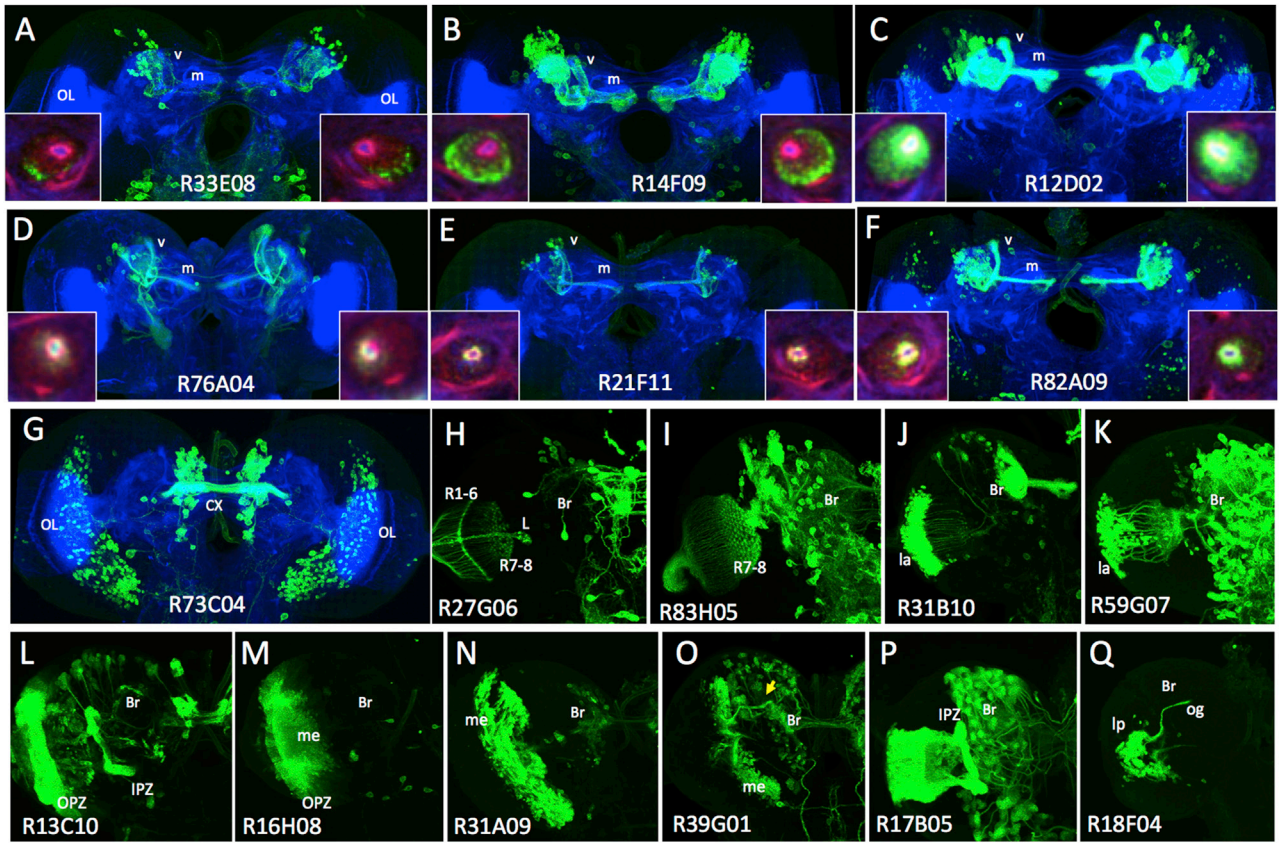

Figure 2. Frontal Views of Larval Brains Showing Expression in Highly Structured Neuropil Regions

(A–F) Different patterns of expression within the mushroom bodies. Insets are higher magnification, optical sections through the right and left peduncles showing distribution of Kenyon cell neurites. The peduncle is stratified with the oldest cells in the periphery and the youngest cells in the central core; each peduncle projects into the z axis of the image from the junction of the medial (m) and vertical (v) lobes. (A) Expression in a small number of the oldest Kenyon cells, situated in the outermost layer of the peduncle and with axons on the outer surface of the lobes. (B) Line expressing in a larger number of these older Kenyon cells. (C) Expression is opposite of (B) with strong expression in the MB neuroblasts and young to moderate aged neurons but missing from the oldest cells. (D) Expression in the MB neuroblasts and the youngest progeny that fill the central core of the peduncle. (E) Similar to (D), but excluding the NBs and the very youngest neurons that occupy the central core. (F) A larger population of neurons with a phenotype like (E).

(G) Larval brain showing highly ordered neurons that will become part of the adult central complex (CX).

(H–Q) Z-projections of confocal stacks of the left-brain hemisphere from various GAL4 lines illustrating expression in a range of optic lobe (OL) cell types. (H) Axons of larval photoreceptors (L) and adult photoreceptors that project to the lamina (R1-6), or to the medulla (R7,8). (I) Only the axons from R7 and R8. (J and K) Two lines with lamina (la) neurons that project into the medulla. (L) Outer (OPZ) and inner (IPZ) proliferation zones. (M) The OPZ along with young medulla (me) neurons. (N) Line with an older age class of medulla neurons. (O) Medulla neurons that project into the central brain (arrow). (P) The IPZ and neurons that will innervate the lobula and lobula plate. (Q) Lobula plate (lp) neurons that project to an optic glomerulus (og).

Green, GFP; blue, N-cadherin; red, neuroglian. The lines vary markedly in their pattern of expression in the central brain (Br).

associated with the neuropils, and cortex glia that wrap the cell bodies (Figures 3A–3D). Besides these standard glia, the larva possesses midline glia, whose numbers expand late in larval life but then largely disappear by the end of metamorphosis (Figure 3E) (Awad and Truman, 1997), and pockets of glia surrounding each immature leg neuropil in the thorax and in discrete locations in the brain (Figure 3F). There are ten lines that drive expression in the cells of the thoracic pockets. Examination of the adult expression patterns of these lines (Jenett et al., 2012) showed one, R10D07, that expressed prominently in adult astrocyte-like glia. Crossing R10D07-GAL4 to a membrane-tagged GFP and then examining nervous systems at various times through metamorphosis showed the adult astrocyte-like glia do indeed arise from the cells in these segmental pockets (Figures 3G–3L). By 12 hr after puparium formation, the cells are migrating out of the pockets and, by 24–30 hr, they have spread over the surface of the neuropil (Figures 3G–3I). Their processes then penetrate deep into the neuropil during the last half of adult development (Figure 3K), generating the mature form of the astrocyte-like glia by the time of adult emergence (Figure 3L).

Spatial Patterns of Expression in the Postembryonic Neurons

The late larval CNS contains tens of thousands of arrested, immature neurons in addition to its functional larval neurons. In the medial region of the brain, these arise from 106 persisting

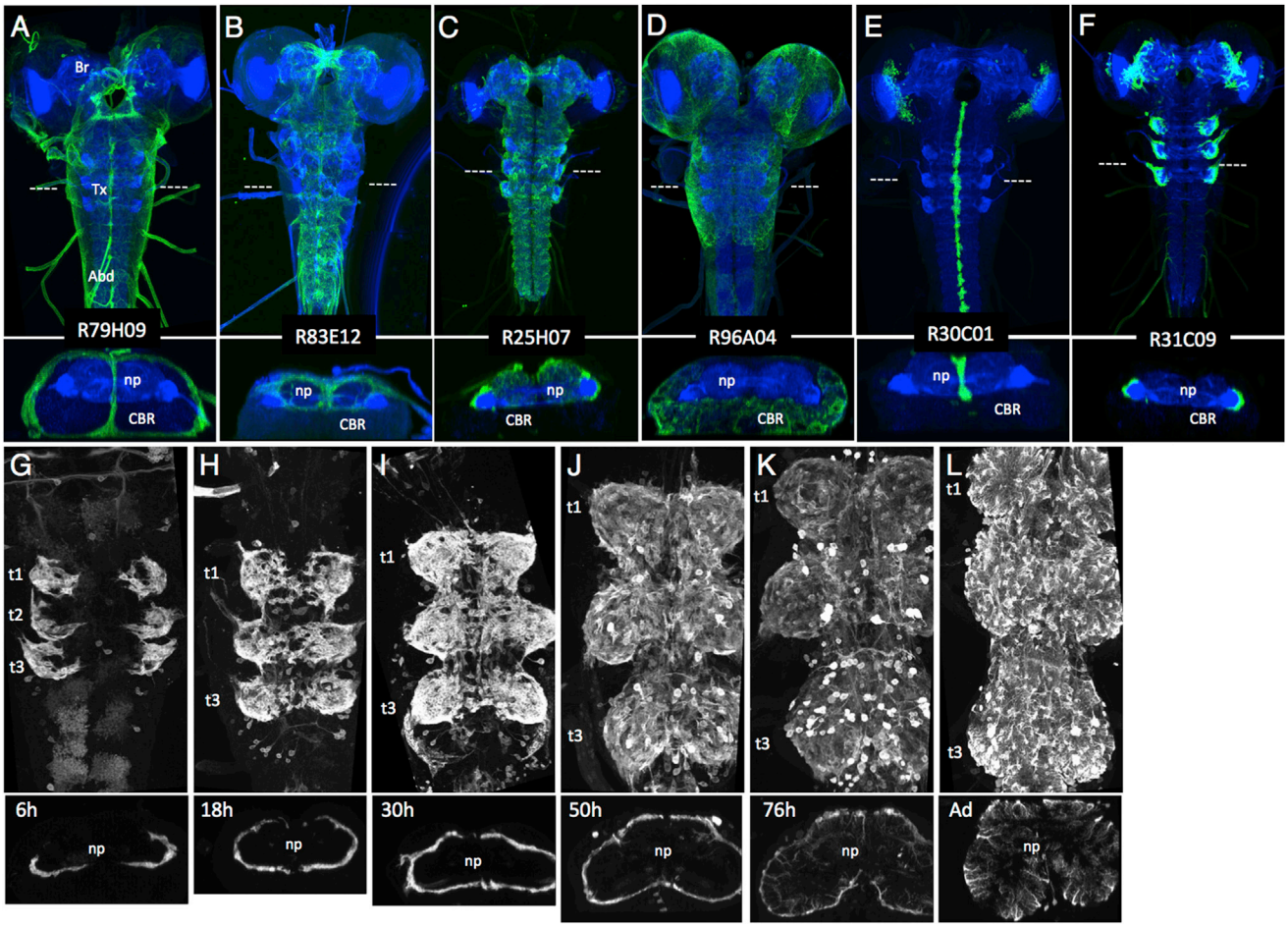

Figure 3. Examples of Glial Expression in the GAL4 Collection

(A–F) Dorsal and cross-section views at the level of T2 (dashed line) of lines that express in (A) perineurial and subperineurial glia, (B) ensheathing glia, (C) astrocyte-like glia, (D) cortex glia, (E) midline glia, and (F) thoracic nests of glial precursors. Abd, abdomen; Br, brain; CBR, cell body rind; np, neuropil; Tx, thorax. (G–L) Dorsal view of the thoracic region of line R10D07 driving GFP expression and showing the migration of glial cells from the pockets around the leg to surround the adult thoracic neuropil. Transverse sections are at the level of t2. Times after puparium formation are approximately (G) 6 hr, (H) 18 hr, (I) 30 hr, (J) 50 hr, (K) 76 hr, as glial processes are invading the neuropil, and (L) newly emerged adult, with highly ramified processes throughout neuropil (np). Green, GFP; blue, N-cadherin; t1–t3, first through third thoracic segments.

neuroblasts per hemisphere (Pereanu and Hartenstein, 2006) and in the VNS they are produced by a basic set of 25 NBs per hemisegment (Truman et al. 2004, Brown and Truman, 2009), with the number varying depending on segment. These immature neurons are in compact clusters, each associated with the parent NB and having one or two axon bundles that extend into the neuropil. In the late larva, isolated immature cells can be distinguished from functional larval neurons because they are small, with scant cytoplasm and have a single, unbranched process that lacks terminal and interstitial arbors. The database identifies the lines that show expression in postembryonic lineages in both the brain and the VNS. We illustrate the utility of this collection for lineage analysis with a preliminary analysis of the thoracic lineages.

In the late larva, the three thoracic neuromeres contain about 2,000 functional larval neurons and about 14,000 immature postembryonic neurons. Despite this numerical disparity, the patterns driven by the CRMs appear to involve the mature neurons more often than the immature ones. We examined a random sample of 259 CRMs that showed sparse expression in thoracic neurons; 75% drove expression in the functional larval neurons, whereas only 40% drove expression in arrested, immature cells. This distribution supports the idea that mature neurons are more diverse in their transcriptional profiles than are arrested, immature cells. The organization of cells showing expression in the two groups was also quite different. For the mature neurons, we typically saw expression in scattered cells that were not obviously related either in terms of potential connectivity or lineage. For the postembryonic neurons, by contrast, clustering was the dominant mode and 62% of our sample showed the immature neurons collected in prominent clusters, each associated with a NB. In 60% of these, we saw only clusters, whereas in the remainder there were a few scattered cells from other lineages in addition to the clusters. This prominent clustering

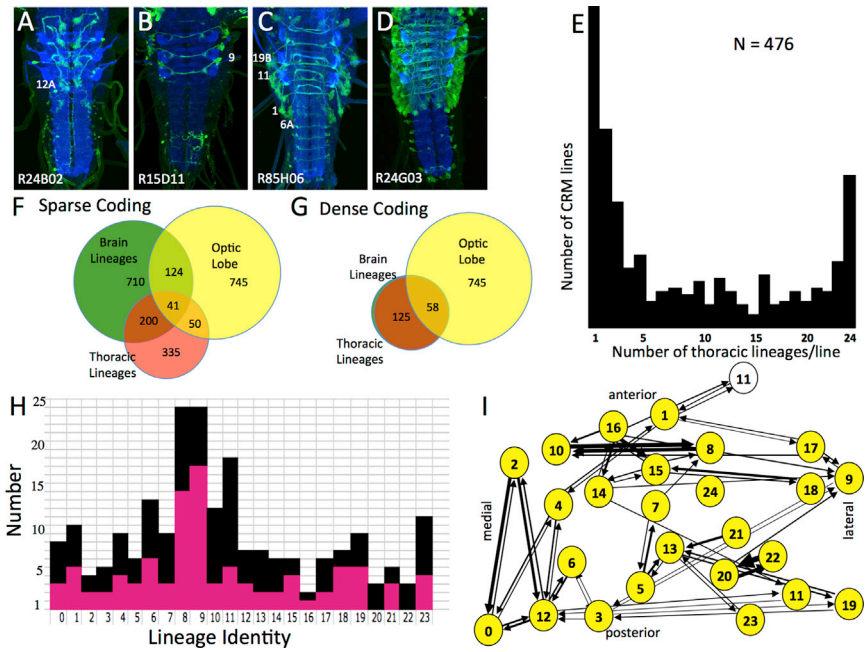

Figure 4. Patterns of Expression in the Post-embryonic Clusters

(A–D) Examples of the range of expression seen in the ventral CNS: (A) single hemilineage (12A) that repeats in segments s2 through t2, (B) full lineage (9) in t1-t3, (C) repeating set of four lineages and hemilineages in thoracic and posterior subesophageal neuromeres, (D) expression by all of the segmental lineages. Green, GFP; blue, N-cadherin.

(E) A distribution of the number of lines that show expression in one to all of the 24 segmental lineages. The U-shaped distribution suggests both sparse and dense patterns control early lineage expression.

(F and G) Venn diagrams showing that virtually all of the CRMs that direct dense expression in the thorax also do so for the brain (G), whereas only about half of those that cause sparse expression in the thorax do so for the brain (F). In neither case was expression in these two regions highly associated with that in the optic lobes.

(H) Coverage of the thoracic lineages by the GAL4 collection is shown by the number of lines that drove expression a given lineage. Based on very sparse lines that drove expression in only one (red) or in two (black) segmental lineages.

(I) Diagram showing the percentage frequency that each lineage coexpresses with the other lineages in a given line. Lineages are depicted according to their position in the hemisegment. We only show coincidence levels over 20%; the width of the lines is proportionate to the frequency of co-occurrence. Based on sparse lines that had two to four lineages expressing per segment.

See also Table S2.

supports the notion that immature cells of the same lineage are transcriptionally similar, but they diverge in their patterns of gene expression as they mature.

The extent of lineage expression ranged from one or a few lineages or hemilineages per hemisegment (Figures 4A–4C) to ubiquitous expression in all of the lineages (Figure 4D). We scored the lineage expression for a sample of 476 lines that represented 55% of lines with expression in the immature clusters. As shown in Figure 4E, the distribution of lines that expressed in various numbers of lineages was U shaped, with the highest frequencies occurring at the two boundaries—either expression in one or a few lineages (or hemilineages) or in most or all of the lineages. We have not done an analysis of the genes in the two categories, but one would expect that the former includes genes involved in establishing lineage identity, whereas the latter includes genes involved in general properties of immature neurons.

There are 335 lines in the total collection that are scored as having sparse expression in the thoracic lineages (defined as five or fewer [<20%] lineages per hemisegment), and 710 lines scored as being similarly sparse in the brain (<20% of the lineages). Sparse lineage expression in the thorax was accompanied by sparse lineage expression in the brain in 60% of the cases (Figure 4F). When we reduced the comparison to the 95 lines that showed single lineage or hemilineage expression in the thorax, 47% of these lines also had expression in brain lineages. The brain expression typically involved more than a single lineage, and we noted no obvious correlation between a given thoracic lineage and specific brain lineages.

At the level of dense expression (>80% of the lineages in a region), there was essentially a complete coincidence of expression in the central brain and the thorax (Figure 4G). Lines that showed dense expression in the thorax also showed dense expression in the brain, and vice versa. However, this extreme overlap in dense expression in these two CNS regions did not carry over into the optic lobes. Dense expression of lineages of central brain was accompanied by dense expression in the optic lobes in only 46% of the cases, and, indeed, in only 60% of the lines was there any type of optic lobe expression. Thus, in the dense expression mode, CRMs almost always regulate expression in both the central brain and VNS but share much less regulatory components with the neurons of the optic lobe.

Expression in Specific Thoracic Lineages and Hemilineages

The extent of coverage of individual thoracic lineages for the lines that showed expression in a single lineage or hemilineage per hemisegment is summarized in Figure 4H. Twenty-two of the 24 major lineages were represented at least once in the sparse subset of the collection. When we included the 59 additional lines that expressed in two lineages per hemisegment, then every lineage was represented at least twice. We did not carry out a similar analysis for the 106 brain lineages, but we expect that this GAL4 collection also has a high level of sparse lineage coverage in the brain.

In the cases in which multiple lineages expressed under the control of a single CRM, we asked if we could detect any underlying pattern of which lineages coexpressed. In the case of the MBs, there are four NBs that generate MB neurons, and they are thought to make identical sets of progeny (Ito et al., 1997). Accordingly, we have not yet found a CRM that consistently

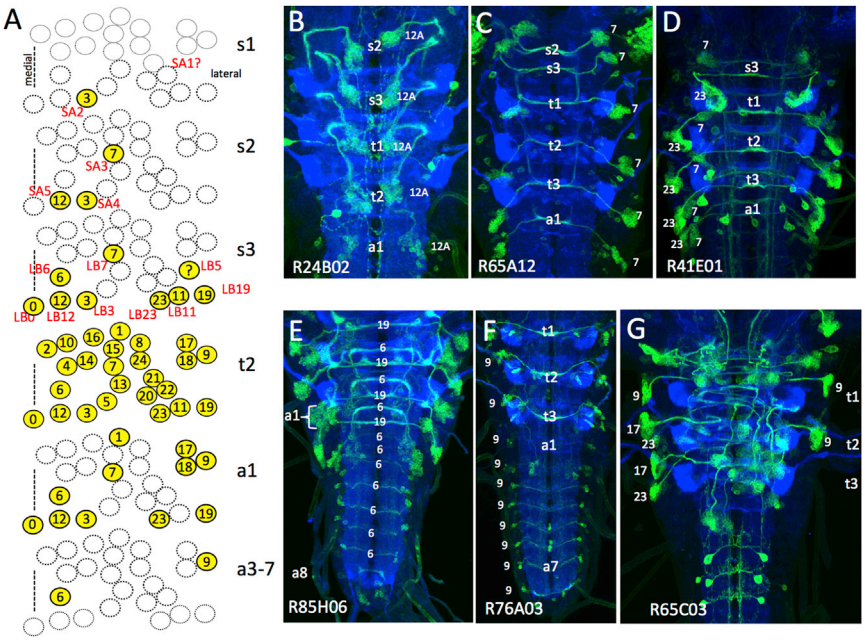

Figure 5. Spatial Aspects of Lineage Expression

(A–F) Use of the GAL4 lines to determine the segmental homologies of lineages found in the subesophageal and abdominal segments.

(A) Map of the canonical set of postembryonic NBs in segment t2 and the loss of specific members in the anterior subesophageal and posterior abdominal segments. The dotted circles are NBs from the t2 set that do not have a postembryonic phase. Yellow NBs are present; numbers identify the NB and/or their lineage. Red names are from Kuert et al. (2014).

(B–D) Examples of lines that allow extension of the series into the subesophageal segments.

(E and F) Examples of lines that allow homologies to be traced through the abdominal segments. a#, s#, t#: abdominal, subesophageal, or thoracic segment.

(G) View of the thoracic region of a line that shows extreme stochastic expression. A set of seven lineages should express in each thoracic hemisegment, but expression occurs randomly within this set in an all-or-none fashion. All examples of the most lateral lineages (9, 17, 23) are labeled for t1 through t3.

drives expression in only a subset of this group of four. Similarly, in the VNS lineages 20 and 22 produce interneurons for the adult leg neuropil, and these two cell groups are difficult to distinguish from one another at either the larval (Truman et al., 2004) or the adult (Harris, 2012) stage. Eighty percent of the sparse lines that directed expression in lineage 20 also drove expression in lineage 22. Similarly, expression in lineages 10 and 8 was linked in almost 80% of the time (Table S2). In this case, though, the two sets of neurons do not share obvious phenotypic characteristics. Figure 4I summarizes the lineages that were found in association with one another at a frequency of 20% or greater in a collection of 115 sparse lines that express in two to four lineages per hemisegment. The overall pattern shows an association bias for lineages in the same medial-lateral row or anterior-posterior column. This pattern mirrors that of fate specification in the early embryo in which there is a grid system set up with some transcription factors, such as *gooseberry* and *engrailed* (McDonald and Doe, 1997), expressed in rows of NBs and others, like *ventral neuroblasts defective* (McDonald et al., 1998), *intermediate neuroblasts defective* (Weiss et al., 1998), and *mesoderm specific homeobox* (Isshiki et al., 1997), expressed in columns. It seems that this early embryonic pattern continues to be reflected in the CRMs that are used during postembryonic specification.

Another pattern that emerged from the analysis of expression came from the hemilineage patterns. Fifty-six of the lines directed expression in thoracic hemilineages, rather than in full lineages. Expression was typically seen in the entire hemilineage (e.g., Figure 4A), but in some cases only a subset of the cells were expressing. In two-thirds of the cases only one hemilineage was involved; 15 lines showed an "A" hemilineages and 22 lines showed a "B" hemilineage. In 19 cases, we found multiple hemilineages in a given line, and, in all but two instances, the hemilineages were of the same type, either all As (six cases) or all Bs (11 cases). The A hemilineage is downstream of *Notch* activation, whereas the B hemilineage is downstream of Notch inactivation. It appears that the 3 Kb average size of the CRM (Pfeiffer et al., 2008) is too small for both pathways to be able to readily act independently of each other.

Segmental Aspects of Lineage Expression

In the embryo, the segmental set of NBs seen in the thorax is almost entirely conserved anteriorly into the subesophageal neuromeres and posteriorly through the abdominal neuromeres (Doe and Goodman, 1985). However, both the subesophageal and abdominal regions show extensive death of NBs late in embryogenesis, and it is difficult to establish the identity of the few survivors that produce the postembryonic clusters. In the abdomen, the number is reduced to only three NB pairs in segments a3 through a7 (Truman and Bate, 1988). In the subesophageal region there are 14 NB pairs for the three gnathal segments (Kuert et al., 2014) as compared to the 73 NB pairs found in the three thoracic segments. Within the thoracic neuromeres, we find that if a given CRM drives expression in a lineage in one thoracic segment, and then it typically drives expression in that lineage in all three thoracic segments (if the lineage is normally present in all three). Out of the 318 sparse thoracic lines in the collection, we found only seven exceptions, and these typically involved lineages that project to dorsal neuropil that shows marked regional specialization within the thorax (Truman et al., 2004; Marin et al., 2012).

For many GAL4 lines, lineage expression was confined to the thoracic segments, but in some lines it extended into the abdominal or subesophageal neuromeres. Such cases provide insights into the lineage identity of the NBs and their clusters in these other regions of the VNS (Figure 5A). For example, enhancers that support expression in postembryonic lineages in both the thorax and abdomen (Figures 5E and 5F) show that lineages 6 (R85H06) and 9 (R76A03) correspond to the abdominal lineages

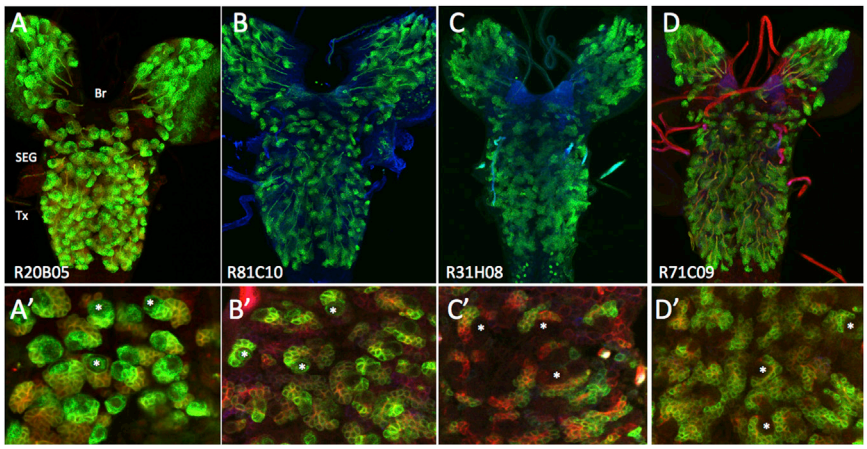

Figure 6. Examples of GAL4 Lines Showing the Range of Cell-type Expression within Lineage Groups

Images are 20 μm projections of the ventral surface of the CNS (X) and a single optical section of the most superficial layer (X′).

(A) All of the members of the postembryonic lineage express GFP from the neuroblast (*) through the oldest members.

(B) Expression in NB, GMCs, and youngest neurons but lacking from the older members.

(C) Reciprocal situation with expression lacking in NB and youngest neurons but present in oldest cells.

(D) Expression in the entire lineage except for the neuroblast.

Green, GRP; red, neuroglian; blue, N-cadherin.

designated vl and dl in Truman and Bate (1988). The lineages in the subesophageal region were recently described (Kuert et al., 2014). For the lineages of the S3 [labial] neuromere, the data from the driver lines support the lineage designations by the authors (Figures 5A–5D), with the exception of their LB5 lineage. In the GAL4 collection, we often find this lineage in isolation (e.g., Figure 1C). When present with thoracic lineages, it is not found consistently with any particular lineage of the thoracic series. Indeed, it appears to be a surviving embryonic NB that is not part of the postembryonic thoracic set (H. Lacin, unpublished data). The more anterior subesophageal lineages could not be assigned with confidence by Kuert et al. (2014) because the array is so reduced, but the GAL4 lines allowed us to assign lineages homologies to all but SA1 (Figure 5A). We did not find the latter lineage in our lines.

Within all of the series, we found no segmental lineage whose expression was consistently linked to expression of particular lineages within the brain. This supports the idea that the NBs of the central brain and of the VNS are not homologous series.

Stochastic Expression Patterns within the Lines

Expression in larval neurons in some lines was stochastic with neurons randomly dropping out of a series on an animal-to-animal basis (e.g., missing cells in a segmental series [Figures 1B and 1E]). We also saw stochasticity in lineage expression, but in this case the unit was the whole lineage (Mellert and Truman, 2012) rather than individual neurons. Seventeen percent of the sparse to moderate lines in the sample that we scored showed stochastic expression of at least some of the lineages. An extreme example is R65C03 (Figure 5G). This line drives expression in seven thoracic lineages, but the chance of any of these lineages expressing is only about 50%, regardless of segment or right-left position. For a given lineage cluster, either all of the cells expressed (including the NB and GMCs) or none of them expressed. In none of the lines did we note stochasticity within a lineage, i.e., a pattern within a given cluster in which there was a random distribution of neurons, some of which expressed GFP and others not. In contrast to the lineages, we saw no instances of all-or-none stochasticity in the hemilineages. Therefore, stochasticity appears to involve a lineage-level decision, most likely at the level of the NB, which then determines the persistent use or nonuse of the CRM in the neurons that are subsequently produced. The results from a transvection study that included stochastic lines indicated that the lack of expression results from an active suppression of the CRM (Mellert and Truman, 2012).

Temporal Aspects of Expression

The collection displays a number of different expression patterns within the context of a lineage cluster. Figure 6A shows line R20B05 that expresses in the complete lineage extending from the NB and GMCs and through the entire cluster of arrested postembryonic neurons. Another common pattern shows expression in the NB, GMCs, and recently born neurons, but then fading out in the older cells of the cluster (Figure 6B). We assume that this pattern reflects an enhancer that is on in the NB and most recent progeny but then shuts off after the young neurons reach their initial targets. We did not find CRMs that resulted in reporter expression in just the NB or the NB and its GMCs. We think that this failure comes from the rapid division times of the NB and the persistence of the mCD8::GFP marker into the daughter cells. We did not test the collection with a GFP that rapidly turns over. There were also patterns that suggested genes being turned on at various times after the cells are born. In R71C09, for example, expression is clearly absent from the NB, but rapidly appears in the GMCs and daughter neurons (Figure 6D). In line R31H08 (Figure 6C), we see a later turn-on with no expression in the NB, GMCs, or young progeny but strong expression in all of the older cells in the cluster.

These arrested, immature neurons are born during the second and third larval instars and they are typically found in the thoracic and subesophageal neuromeres, with only three small, paired clusters in the abdomen. Of the lines that showed expression in postembryonic clusters at the start of metamorphosis, about half showed little or no expression in corresponding regions at hatching, a result that would be expected because postembryonic neurogenesis had not yet begun. In the remainder, though, there was robust expression in similarly placed clusters of neurons, typically in both the thoracic and abdominal neuromeres (Figure 7). The R19B03 line that contains an enhancer element for *msh* illustrates the reason for this pattern. This transcription factor specifies the most lateral columns of neuroblasts that

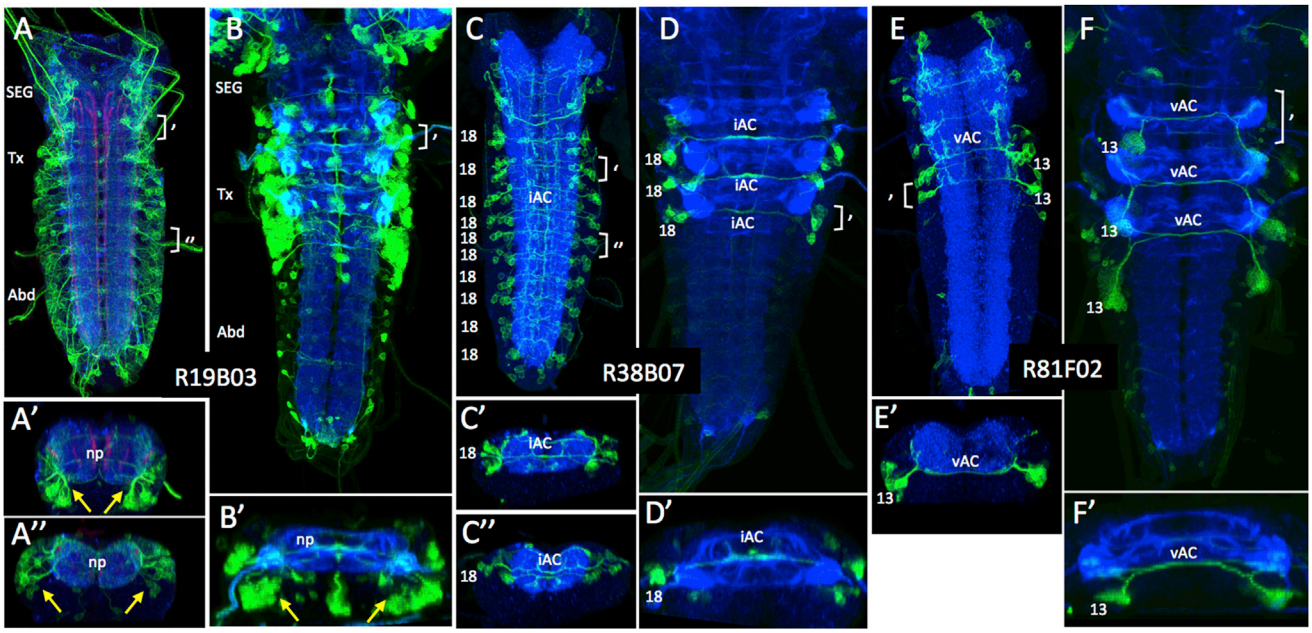

Figure 7. Comparison of Lineage-Related Expression in Larval Nervous Systems at Hatching and Just before Metamorphosis

Confocal projections of the VNSs of L1 (A, C, and E) and L3 (B, D, and F) larvae from lines that show lineage-restricted expression in the young neurons in the respective lineages. Shown are dorsal projections (X) and transverse projections at the level of the thorax (X′) and the abdomen (X″).

(A–D) In lines R19B03 and R38B07 expression is the L3 is largely restricted to the specific postembryonic clusters in the thoracic segments (B and D). Those cells have not yet been born at the time of hatching (A and C), but their embryonic-born counterparts are still young enough to express, including the abdominal homologs that have no postembryonic proliferation phase.

(E and F) Line R81F02 expresses in both the embryonic-born (E) and postembryonic-born (F) members of the lineage 13, but expression is confined to only the thoracic lineages in both cases. Green, GFP; blue, N-cadherin; Abd, abdomen; iAC, intermediate ventral commissure; np, neuropil; SEG, subesophageal ganglia; Tx, thorax; vAC, ventral anterior commissure. Numbers identify the lineage clusters.

make cells residing in the dorsolateral region of the CNS (Isshiki et al., 1997). In the third instar larval CNS, these include the secondary neurons in lineages 8, 15, 16, 17, 18, and 20–23 as well as the median lineage (lineage 0). This expression is seen in the postembryonic clusters from the subesophageal neuromeres through segment a1. These postembryonic-born cells are not present at hatching, but in their place we find expression in clusters of embryonic-born neurons in the corresponding dorsolateral domain. Interestingly, in the hatchling the pattern extends through all of the abdominal segments. We assume that these neurons are the embryonic-born members from the same lineages that we see in last stage larva. Their NBs all have an embryonic neurogenic phase in all segments, but only the thoracic and subesophageal NBs have a postembryonic phase. We expect that *msh* expression is restricted to immature and young neurons of the respective lineages so that its expression fades once the cells are mature. Consistent with this interpretation, we find that this enhancer drives essentially no expression in the ventral CNS of the adult. Line R38B07 presents a similar story for a single lineage, lineage 18 (Figures 7C and 7D), and R81F02 marks the embryonic (Figure 7E) and postembryonic (Figure 7F) members of lineage 13. The curious feature of the latter is that we fail to see abdominal members at either time.

None of the above three lines showed any lineage-related expression in the adult CNS. This lack of lineage expression through metamorphosis appears to be a general phenomenon, although we have noted rare exceptions in the collection. Nevertheless, if needed, the larval pattern can be used in conjunction with flip-out strategies to preserve lineage expression into the adult stage (e.g., Harris, 2012).

Conclusions

This collection of GAL4 lines provides an extensive and versatile resource for work on the larval nervous system of *Drosophila*. Compared with expression of the same lines in the adult (Jenett et al., 2012) the larval versions include fewer neurons, and many lines are sparse enough for meaningful behavioral studies (e.g., Vogelstein et al., 2014). In addition, larval data make these lines valuable for developmental studies because metamorphosis has been used to examine phenomena related to neurogenesis, cell death, and cellular remodeling.

EXPERIMENTAL PROCEDURES

Larval Dissection and Immunohistochemistry

Larval tissues were dissected in phosphate buffered saline (PBS) and fixed for 1–2 hr in 4% paraformaldehyde at room temperature. After multiple rinses in PBS with 1% Triton X-100 (PBS-TX), tissues were mounted on poly-L-lysine (Sigma-Aldrich)-coated coverslips and then transferred to a coverslip staining jar (Electron Microscopy Sciences) with blocking solution, 3% normal donkey serum in PBS-TX for 1 hr. After blocking, tissues were incubated with mouse antineuroglian (1:50 BP-104; Developmental Studies Hybridoma Bank), rat anti-N-cadherin (1:50 DN-Ex #8; Developmental Studies Hybridoma Bank),

and rabbit anti-GFP immunoglobulin (Ig) G (1:1,000; Invitrogen A11122) in PBS-TX and incubated 2 days at 4°C. After multiple rinses in PBS-TX, tissues were incubated 2 days at 4°C in the cocktail of secondary antibodies containing Alexa Fluor 568 Donkey anti-mouse IgG (1:500; Invitrogen), Alexa Fluor 647 Donkey anti-rat IgG (1:500; Jackson ImmunoResearch), and fluorescein FITC-conjugated Donkey anti-rabbit (1:500; Jackson ImmunoResearch). Nervous systems were then washed two to three times in PBS-TX, dehydrated through a graded ethanol series, cleared in xylene, and mounted in DPX (Sigma).

Immunolabeled larval nervous systems were imaged on a Zeiss 510 Confocal microscope using their 40× oil immersion objective (numerical aperture 1.3). Images of each nervous system were assembled from a 2 × 3 array of tiled stacks, with each stack scanned as an 8 bit image with a resolution of 512 × 512 and a Z-step interval of 2 μm. Unlike the adult brain (Jenett et al., 2012), there is not a standard template of the larval CNS to which all of the images could be registered.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.065.

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