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Developmentally regulated expression of reporter gene in adult brain specific *GAL4* enhancer traps of *Drosophila melanogaster*

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Introduction

The genetic regulation of adult brain development has remained enigmatic to developmental biologists since long. The practical difficulty in understanding the adult brain development is that most of the mutational searches done for genes in different model systems yield mutations inducing embryonic lethality, making it impossible to assess their role in adult development. In *Drosophila*, enhancer trap tool has emerged as an answer to this problem. In this paper we present the preliminary results of a P-*GAL4* enhancer trap screen. About 20 previously generated adult brain specific P-*GAL4* enhancer trap strains have been assayed for reporter expression in brain during different larval instars and the results are presented. The observations are significant for two reasons. The expression patterns of the reporter indicate that the native genes with P-insertion in these strains should have a developmental role. Each strain is a potential identification of a gene with possible function in brain development. Since the enhancer traps reported here are of the *GAL4*-UAS type, they will also be useful for targeting the expression of any gene of interest specifically to different cell types of the developing *Drosophila* brain.

Nervous system of an organism, consisting of millions of neurons specialized to carry out specific functions in an extraordinarily coordinated manner, depicts the highest order of complexity with respect to its structural and functional organization. The development of this complex nervous system requires generation of large number of neurons from a small initial quota of precursor cells, their fate specification, differentiation, establishment of proper connectivity amongst themselves as well as their target cell types, leading to

the formation of specialized functional circuits. Understanding the genetic and molecular basis of the events involved in constructing this higher order system has been a long lasting challenge, more so with the development of adult brain. *Drosophila*, with its battery of tools available for genetic manipulations, has been proved to be an unparalleled model system to address the questions of genetic interactions and pathways regulating development and differentiation.

A cumulative effort of several researchers has given us a neuroanatomical and cell lineage based fair picture about the developing larval brain (Hartenstein *et al.* 2008), anterior posterior regionalization (Lichtneckert and Reichert 2008), dorsoventral differentiation (Urbach and Technau 2008), development of adult olfactory system (Rodrigues and Hummel 2008) and optic lobes (Fischbach and Hiesinger 2008) in *Drosophila*. However, the knowledge that we have about the molecular players that regulate these highly orchestrated processes is meagre. Several genes have been identified as involved in different aspects of *Drosophila* adult brain development: genes such as *mushroom body defective (mud)*, *anachronism (ana)*, *terribly reduced optic lobe (trol)*, *baboon (babo)*, *eyeless*, *minibrain (mnb)*, *wingless (wg)*, *DEcad*, *Grainy head*, have been shown to be involved in larval brain neuroblast proliferation (Hartenstein *et al.* 2008). Homeobox containing genes such as *orthodenticle* and *empty spiracle* are implicated in anterior/posterior specification of the developing brain (Lichtneckert and Reichert 2008). *Atonal* and *robo* expressions are needed for establishing specific connectivity in the olfactory lobe (Jhaveri and Rodriguez 2002; Jhaveri *et al.* 2004). A comparatively greater number of genes taking part in optic lobe development has been uncovered (Fischbach and Hiesinger 2008). Despite these successful studies, owing to the complexity of the process, we are still at infancy of our understanding of the molecular networks operative during adult brain development in *Drosophila*. There is a need to further our investigations for

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identification and isolation of genes that might have critical role in brain development. Here, we present preliminary results of a screen done towards this goal.

We have employed the enhancer trap system (O’Kane and Gehring 1987; Bellen *et al.* 1989; Wilson *et al.* 1989) which serves as a potential means to identify and isolate new genes based on their expression pattern, thus enabling us to overcome the probable embryonic lethality that the classical mutational screen would result in. P-*GAL4* system is a second generation enhancer trap system (Brand and Perrimon 1993), which was evolved as a tool to achieve targeted ectopic expression of genes in *D. melanogaster*. A previous screen (Shyamala and Chopra 1999) had yielded 20 *GAL4*-enhancer trap strains which have reporter gene expression in the adult brain. Each of these strains has now been analysed for the reporter expression in brain at different larval instars. Since the reporter gene expression has been shown to be in close match with the expression of the native gene at the site of P-insertion (Wilson *et al.* 1989), larval expression patterns of reporter gene in these P-*GAL4* insertion strains strongly suggests probable developmental role for the respective native genes.

Materials and methods

Fly stocks

Transgenic lines containing random insertion of P-*GAL4* with adult brain specific *GAL4* expression (Shyamala and Chopra 1999) and transgenic line with UAS Nuc *lacZ* on second chromosome.

Histochemical detection of activator gene expression

Virgins were collected from w-;UAS Nuc *lacZ* reporter stock and were crossed to each of the P-*GAL4* transgenic strains. The F₁ embryos were collected within a hours time window, staged as 0 h at hatching, and the different instars were staged from that time point onwards. The entire central nervous system (CNS) was dissected out from the staged larvae in phosphate buffered saline (PBS). The CNS was immediately fixed in 4% paraformaldehyde prepared in PBS. The fixed CNS was then washed in PBS and stained in X-gal staining solution (0.060 mL 5% X-gal, 0.020 mL 100 mM potassium ferricyanide, 0.020 mL 100 mM potassium ferrocyanide, 0.050 mL 1 M sodium phosphate buffer pH 8.0, 0.850 mL 1× PBS) by incubating it at 37°C in a moist box in dark. The stained brains were mounted in 70% glycerol, observed and imaged with Olympus BX60 microscope (Olympus Corporation, Tokyo, Japan).

Results and discussion

Twenty P-*GAL4* enhancer trap strains that had reporter gene activity in the adult brain were crossed to UAS *LacZ* line, and the F₁ larval neural ganglion were stained for β -galactosidase

activity at different instars. A remarkable fraction of these, amounting to 15 strains showed *LacZ* activity in the larval ganglion in a spatiotemporal specific pattern. The expression patterns in different strains will be discussed on a broad classification based on the gross area of reporter expression.

Optic lobe

Optic lobes are the largest functional compartments of the adult *Drosophila* brain. The precursor cells can be identified as two major clusters: the inner optic focus and the outer optic anlagen. The earlier studies of developing optic lobes have revealed very interesting and crucial molecular interactions between the developing eye and the laminar primordial (Huang and Kunes 1996; Huang *et al.* 1998). In the present study, we have three strains which show reporter activity in the optic region. SG21.1 at third instar stage shows moderately distributed cells with reporter activity at the outer optic anlagen (figure 1k). The expression is visible in 3–4 clusters of cells at the outer optic anlagen area in the mid-second instar ganglion of SG29.1 (figure 2g). This expression is withdrawn and becomes undetectable by the third instar stage (figure 2h). More interesting expression is seen in case of SG26.1, which becomes visible in the first instar brain. It is seen as two clusters of cells one in each brain hemisphere towards the optic precursor region (figure 2d). By third instar stage, very strong expression is seen in the inner optic focus which extends laterally as a stripe towards the outer optic anlagen (figure 2f).

Olfactory lobe and mushroom body

Olfactory or antennal lobes are the primary centres for processing the odour cues from peripheral systems. The olfactory sensory neurons synapse with interneurons in anatomically recognizable units called glomeruli within the olfactory lobe. Mushroom bodies (MB) which are situated dorsal to the olfactory lobes are complex structures that function as higher order association and storage centres of olfactory learning and memory, and are often compared to the cerebral cortex of higher mammals (de Belle and Heisenberg 1994; Davis and Han 1996; Tettamanti *et al.* 1997). We have four strains which express reporter gene activity in the general anatomical location of the olfactory lobe and mushroom body precursor cells. In SG21.1 (figure 1e) during second instar stage, a pair of compact clusters of cells placed in the MB region exhibit reporter gene activity. Each of these clusters by third instar stage gets clearly demarcated into two distinct groups with a more pronounced level of reporter activity (figure 1k). SG1.1 (figure 1b) and SG31.1 (figure 1l) strains show a very strong expression of *LacZ* in the MB/olfactory region of the third instar larval brain, where as it is relatively moderate expression in case of SG29.1 third instar stage (figure 2h). In all these strains, the indication of reporter expression becomes visible in early second instar brain.

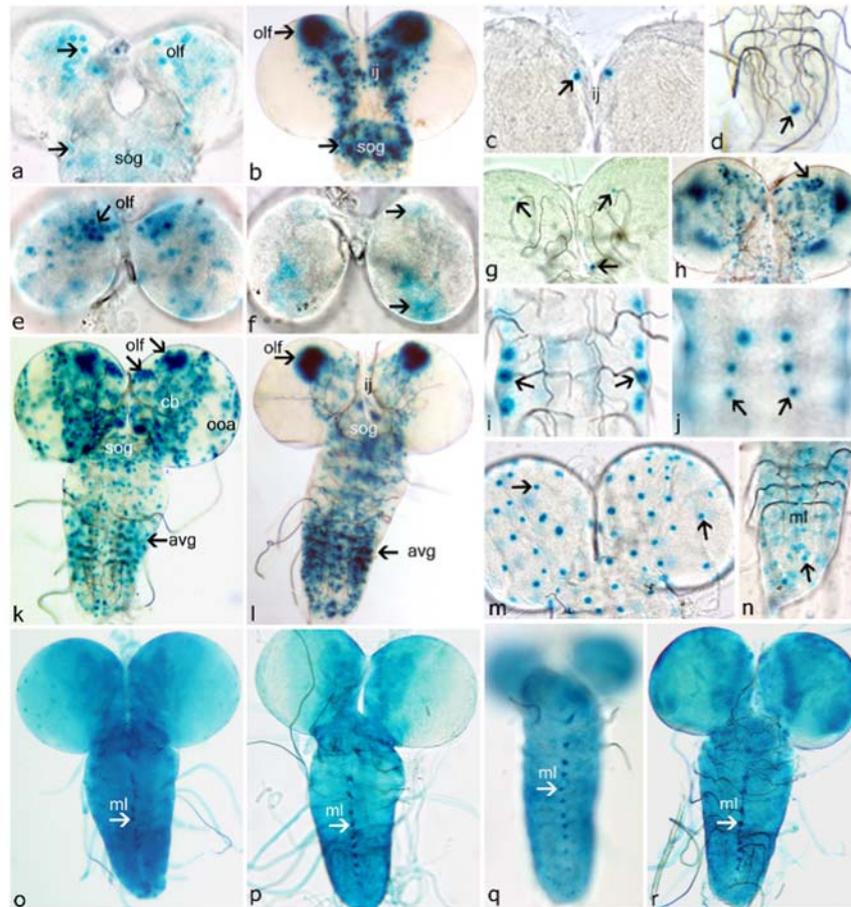


Figure 1. *GAL4* expression pattern in the larval CNS of representative adult brain specific *GAL4* enhancer trap strains. Transgenic strains with P-*GAL4* insertion were crossed to UAS-Nuc *LacZ* strain and the F₁ larval ganglion at different instars was stained for β -galactosidase activity. (a) Second instar, (b) third instar of SG1.1 where the reporter expression appears at 2nd instar stage and becomes strong in the olfactory/mushroom body region (olf), interhemispheric junction (ij) and the suboesophageal region (sog) by third instar. (c & d) Third instar larval brain of SG19.1 showing specific reporter activity in a pair of cells at the anterior interhemispheric junction (c) and a pair of compactly placed cells at the posterior end of the ventral ganglion (d). (e) Second instar larval ganglion of SG21.1. The cells with reporter activity appear as single group in the olfactory (olf) region of each brain lobe. (f) Second instar larval brain of SG31.1. Reporter expression is first seen in the optic and olfactory precursors (arrows). (g) Third instar larval brain of SG34.1, with specific expression in a pair of cells at the central brain (cb) region and a single unpaired cell situated at the right side of sub oesophageal ganglion (sog). (h) Third instar brain of SG 33.1 showing strong β -galactosidase activity in large clusters of cells in the brain hemispheres. (i & j) Ventral ganglion of the third instar brain of SG13.1, with reporter gene expression in four pairs of large marginal (i) and three pairs of large medially (j) placed cells. (k) Third instar larval ganglion of SG21.1. The cells with reporter activity appeared as single group in the olfactory (olf) region of each brain lobe during second instar (e), but are seen as two distinct clusters by third instar (arrows) stage. Moreover, the third instar brain shows moderately distributed cells in the central brain (cb) outer optic anlagen (ooa), sub oesophageal ganglion (sog) and the anterior abdominal neuromeres (avg) of the ventral ganglion with reporter expression. (l) Third instar larval brain of SG31.1. Reporter expression is first seen in the optic and olfactory precursors (arrows) in the second instar (f), and then becomes remarkably strong in the olfactory (olf) region and the anterior abdominal neuromeres (avg) of the ventral ganglion by third instar. Moderately distributed reporter expressing cells are also seen in the central brain, suboesophageal regions. Anterior abdominal neuromeres (avg) show strong reporter expression. (m) Third instar larval brain of SG9.1 with strong β -galactosidase activity in scattered cells throughout the larval brain. (n) Second instar larval brains of SG16.1. The reporter expression is seen in cell clusters along midline. (o) Third instar larval brains of SG16.1. The reporter expression that is seen in cell clusters along midline in second instar (n) now becomes confined to single row of cells along the midline (ml). The brain in this strain also shows an overall diffuse reporter activity at third instar stage. (p) Third instar brain of SG28.1 with a rather diffuse reporter expression globally but relatively strong in the midline glial cells (ml). (q) Second instar, and (r) Third instar larval brains of SG5.1 showing strong reporter gene activity in the mid-line glia (ml), besides a general diffuse activity through out.

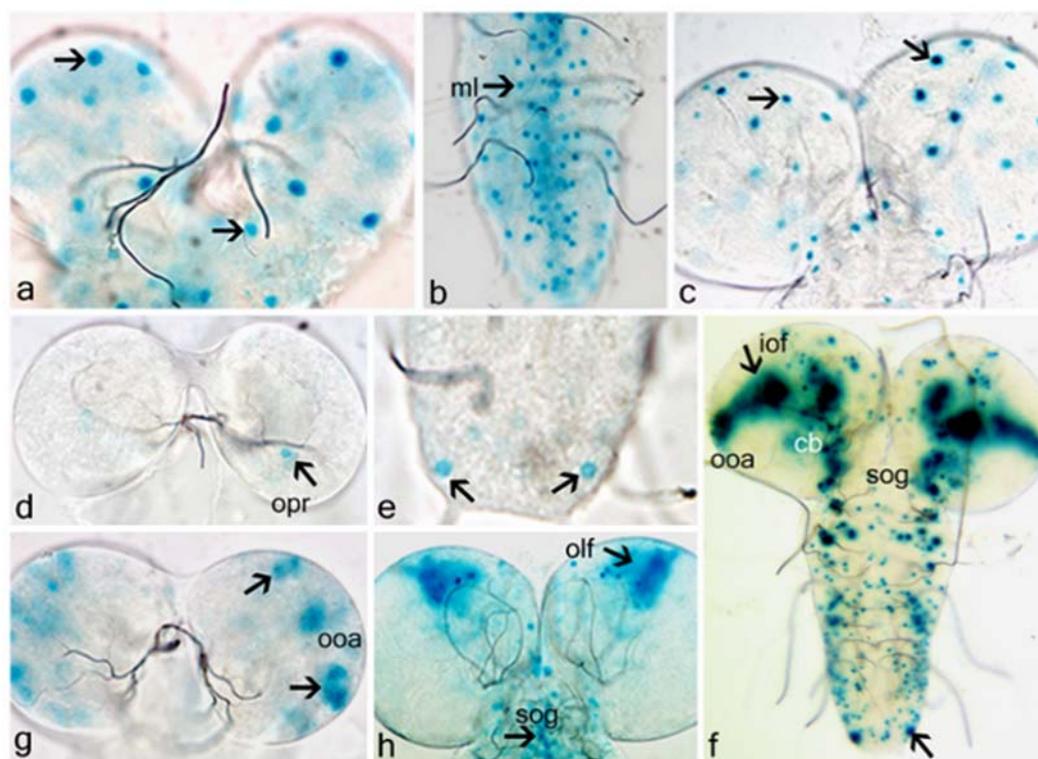


Figure 2. *GAL4* expression pattern in the larval central nervous system of representative adult brain specific *GAL4* enhancer trap strains. Transgenic strains with P-*GAL4* insertion were crossed to UAS-Nuc *LacZ* strain and the F₁ larval ganglion at different instar was stained for β -galactosidase activity. (a & b) the brain lobes and ventral ganglion, respectively, of the second instar larval neural ganglion of SG15.1. Large cells distributed throughout the brain lobes (a) show reporter expression. Ventral ganglion (b) shows a characteristic pattern of reporter gene expression with expression in doublets of cells arranged on either side of the midline (arrow). (c). Third instar brain lobes of SG17.1 with reporter activity in large cells distributed throughout. (d & e) Second instar larval brain of SG26.1. Very faint expression is seen in small clusters in the brain lobes (d), whereas a pair of large cells situated at the posterior end of the ventral ganglion (e-arrows) show strong expression of the reporter. (f) Third instar larval brain of SG26.1. Strong expression is seen in the inner optic focus (iof) which extends outwards to the outer optic anlagen (ooa). Clusters of cells in the central brain (cb) and the suboesophageal ganglion (sog) have strong reporter expression. Moderate levels of expression are seen along the margin of the ventral ganglion. The pair of cells at the posterior end of the ventral ganglion (arrow) continues to have high levels of reporter gene activity. (g) Second instar brain lobes of SG29.1. Expression is seen in a small cluster in the olfactory region and 2–3 large clusters in the optic anlagen (ooa). (h) Third instar brain lobes of SG29.1. Expression is withdrawn from the optic region, whereas it becomes stronger in the olfactory region (olf). Few cells at the suboesophageal region (sog) also show β -galactosidase activity.

Central brain, inter hemispheric junction and suboesophageal ganglion

Central brain is a complex of four distinct neuropile structures namely, the protocerebral bridge, fan-shaped body, noduli and the ellipsoid body. It is a median unpaired complex which occupies the interhemispheric junction (IJ) of the adult brain (Boquet *et al.* 1999). It is involved in integrating the two hemispheres of the brain functionally and has an influence on the larval and adult locomotory behaviour (Barth and Heisenberg 1997; Martin *et al.* 1999). This central complex develops from the cells located at the interhemispheric junction of the larval brain. The suboesophageal ganglion (SOG) is the region that receives terminals of sensory neu-

rons form mouth and the surface of the head capsule, and the dendrites of the motor neurons that supply the head muscles. Strain SG1.1 (figure 1b) shows strong expression of the reporter in the IJ and the SOG region of the third instar larval brain. Moderately distributed cells over a slightly broader area at IJ and SOG exhibit reporter gene activity in case of SG 21.1 (figure 1k) and SG31.1 (figure 1l). SG26.1 third instar brain has rather distinct and compact clusters with strong reporter expression in the central brain region (figure 2f)

Ventral ganglion and midline glia

Ventral ganglion (VG), sometimes not considered as brain in adult *Drosophila*, is the posterior part of the CNS which basically represents fused thoracic and abdominal neuromeres.

Its primary role is associated with the peripheral sensory and motor functions of the thoracic and abdominal segments. In the present screen, we have found many strains which show reporter gene expression in the VG. SG21.1 (figure 1k) and SG31.1 (figure 1l) have moderately dispersed cells with reporter gene activity through out the ventral ganglion. Reporter gene expressing cells are seen at high density in the anterior abdominal neuromeres. SG26.1 shows numerous cells along lateral margin with reporter gene activity (figure 2f). SG13.1 has a more specific pattern, where four pairs of marginally placed large cells (figure 1i), along with another set of three pairs of cells located medially (figure 1j), with strong reporter activity are seen at the anterior abdominal neuromeres.

In SG15.1 (figure 2b) distinct pattern is seen with cells arranged as doublets on either side of the midline showing reporter expression. SG5.1 (figure 1r), SG16.1 (figure 1o) and SG28.1 (figure 1p) third instar brains, though have a more or less diffuse pattern of staining through out, the midline glial cells show much stronger reporter activity.

Global pattern

Some of the strains like SG9.1 (figure 1m), SG17.1 (figure 2c), SG15.1 (figure 2a) have large isolated cells more or less uniformly scattered through out the CNS exhibiting strong reporter gene activity. The native genes in these P-insertion strains might be involved in regulating more general processes in relation to the neuroblasts.

Specific isolated cells pattern

SG19.1 has a highly specific pattern of reporter gene expression (figure 1,c&d). Two large neuroblasts situated at the anterior end, towards the interhemispheric junction show strong expression. These cells probably are precursors of the ocellar neurons because in this strain we see expression in the ocellar nerve and the ocellar neuron of the adult brain (Shyamala and Chopra 1999). Added to this, there is a pair of very closely placed cells medially located at the posterior end of the ventral ganglion which has strong reporter expression. SG34.1 (figure 1g) has a pair of cells at the anterodorsal region of the central brain, and a single cell situated specifically at the right side of the suboesophageal ganglion, showing reporter activity.

The larval brain in *Drosophila*, besides having the functional larval neurons, also lodges a population of neuroblasts. They do not contribute to the functional component of the larval brain as such but re-enter into mitotic phase to result in immature post mitotic neurons (Truman 1990). These neurons, referred to as adult specific neurons, undergo extensive reorganization during metamorphosis to form fully differentiated adult functional neurons. The cells which show reporter gene expression in most of the P-*GAL4* strains described here are large and round in shape indicating strongly that we are looking at the adult specific neuroblasts/immature neurons. In an enhancer trap system, it has been shown that

the expression of the reporter gene will be similar to that of the native gene at the site of P-insertion, as both will be driven by the same genomic enhancer element (Wilson *et al.* 1989). The present observation implies that the random P-insertions in these strains have mostly marked native genes which are expressed in adult brain precursors residing in the larval neural ganglion. Such a developmentally regulated expression of a gene in turn would indicate a probable developmental role for the native gene. Further characterization of these strains with respect to localization of the P insertion and identification of the native genes is under progress.

As each of the P-*GAL4* strains isolated in this screen has specific spatial and temporal pattern of expression of *GAL4*, they can be efficiently used to target the expression of a gene of interest to definite domains/cells of the larval brain, by having the gene of interest cloned downstream of a *GAL4* specific UAS sequence (Brand and Perrimon 1993).

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