

Neuroblast Ablation in *Drosophila* P[GAL4] Lines Reveals Origins of Olfactory Interneurons

Reinhard F. Stocker,¹ Gertrud Heimbeck,¹ Nanaë Gendre,¹ J. Steven de Belle²

¹ Institut de Zoologie, Université de Fribourg, Pérolles, CH-1700 Fribourg, Switzerland

² Max-Planck Institut für Biologische Kybernetik, Spemannstr. 38, D-72076 Tübingen, Germany

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ABSTRACT: Hydroxyurea (HU) treatment of early first instar larvae in *Drosophila* was previously shown to ablate a single dividing lateral neuroblast (LNb) in the brain. Early larval HU application to P[GAL4] strains that label specific neuron types enabled us to identify the origins of the two major classes of interneurons in the olfactory system. HU treatment resulted in the loss of antennal lobe local interneurons and of a subset of relay interneurons (RI), elements usually projecting to the calyx and the lateral protocerebrum (LPR). Other RI were resistant to HU and still projected to the LPR. However, they formed no collaterals in the calyx region (which was also ab-

lated), suggesting that their survival does not depend on targets in the calyx. Hence, the ablated interneurons were derived from the LNb, whereas the HU-resistant elements originated from neuroblasts which begin to divide later in larval life. Developmental GAL4 expression patterns suggested that differentiated RI are present at the larval stage already and may be retained through metamorphosis. © 1997 John Wiley & Sons, Inc. *J Neurobiol* 32: 443–456, 1997

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INTRODUCTION

The antennal lobe (AL) is the primary association center for smell in insects. In *Drosophila melanogaster*, the AL collects the entire set of olfactory projections both from the antenna and from the maxillary palps (Stocker et al., 1983; Singh and Nayak, 1985; Charro and Alcorta, 1994; Stocker, 1994). The major targets of these afferents are two types of AL interneurons, local interneurons [LocI (intrinsic neurons)] and relay interneurons [RI (projection neurons)], elements which are known from a number of insect species. LocI establish ar-

borizations in most, or perhaps all of the glomeruli of the AL [cf. Fig. 6(A)]. In *Manduca sexta*, LocI were claimed to inhibit output interneurons in the absence of olfactory input (Christensen et al., 1993), while in another moth, *Spodoptera littoralis*, only excitatory responses were observed (Anton and Hansson, 1995). RI collect information from single or few glomeruli and provide the links with two higher brain centers, the mushroom body (MB) calyx and the lateral protocerebrum (LPR) [cf. Fig. 6(A)]. From studies in moths, no simple functional definition of RI is possible, since units responding to single odor components as well as those transmitting integrated odor signals have been observed (Anton and Hansson, 1995). This suggests that odor processing is not restricted to the AL, but continues in higher brain centers.

The adult AL of *Drosophila* is derived from a larval precursor, which is probably the unique target of larval olfactory afferents (Stocker et al., 1995; Tissot et al., 1997). Since the chemical environment

Correspondence to: R. F. Stocker (e-mail: Reinhard.Stocker@unifr.ch)

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of the creeping larva and of the flying insect differ considerably, the AL has to fulfil different olfactory tasks in the two subsequent periods of life. How it adapts to the new requirements is not understood, but it is obvious that substantial reorganization must occur during metamorphosis, concomitant with the replacement of the olfactory sensilla. At the interneuron level, three processes are conceivable: the degeneration of larval neurons, their persistence, or the formation of new adult-specific neurons (Truman, 1990). Persisting larval neurons were estimated to make up no more than 7% of the cells in the adult thoracic central nervous system (CNS), most of which are motor or modulatory (Truman and Bate, 1988). Adult-specific cells are primarily interneurons devoted to the processing of adult sensory information (Truman, 1990). In *Drosophila*, structural changes in the AL are manifest by a 50–100-fold increase in volume, by the transition from an apparently homogeneous larval AL to a glomerular adult AL, and by a high level of cell proliferation in the AL cortex with a peak in the late third larval instar (L3) (Stocker et al. 1995; Tissot et al., 1997).

Experimental evidence on the development of the AL comes from a recent study in which the DNA-synthesis inhibitor hydroxyurea (HU) (Timson, 1975; Truman and Booker, 1986) was applied at the early first instar larva (L1). At this stage, only five pairs of neuroblasts are dividing in the CNS: four in the dorsal brain and a single pair in the lateral brain. This treatment leads to a near complete loss of both the larval and adult MBs, and to a volume reduction in the AL of about 30% (de Belle and Heisenberg, 1994), consistent with evidence that the dorsal neuroblasts give rise to the cellular elements of the MB (Prokop and Technau, 1991; Ito and Hotta, 1992), whereas the lateral neuroblast (LNb) is the founder cell of many interneurons in the AL (Stocker et al., 1995). However, tagging of dividing cells during successive developmental stages with bromodeoxyuridine suggests that many AL interneurons derive from other lineages (Stocker et al., 1995). After the early onset of proliferation of the LNb, up to a dozen other neuroblasts begin to divide in the late L1 and the early second instar (L2). All of them remain mitotically active until the early pupa, when proliferation abruptly ends.

Previous HU studies did not permit an assignment of the different types of interneurons to particular cell lineages (de Belle and Heisenberg, 1994). Yet, owing to enhancer trap technology (O’Kane and Gehring, 1987; Brand and Perrimon, 1993), efficient cellular markers can now be generated which facilitate the analysis of development in iden-

tified cell types, especially in the nervous system. In a screen for P insertion lines that express GAL4 in the chemosensory system, we have isolated several lines which label specific types of AL interneurons as visualized by *tau* reporter gene expression. This provides perfect resolution of axonal processes, owing to the ectopic expression of the microtubule-associated TAU protein. In GAL4/UAS-*tau* transheterozygotes, line GH146 showed a highly selective expression in the RI, whereas line GH298 labeled LocI. Intriguingly, RI expressed GAL4 in line GH146 continuously from the second larval moult until adulthood, which suggests that these cells may be retained through metamorphosis. HU ablation in these lines at different larval intervals revealed the neuroblast of origin of these interneurons and/or the time at which they were born. We show that LocI and a subset of RI derive from the early dividing LNb, whereas the remaining elements are formed by neuroblasts that begin to divide later in larval life.

MATERIALS AND METHODS

Strains

Flies of the *Canton-S* strain and of the recently isolated homozygous P[GAL4] insertion lines GH146 and GH298, crossed to either UAS-*lacZ* (Brand and Perrimon, 1993) or to UAS-*tau* (K. Ito and S. Schneuwly; stocks kindly provided by T. Raabe, University of Würzburg) were used for the experiments. The *tau* constructs used do not show any autonomous TAU expression in the adult CNS.

Collection of Timed Larvae and Pupae

Females were allowed to lay eggs for 1 h in small petri dishes containing food and fresh yeast. Timed larvae were obtained by checking larval hatching every 1 h from 20 h after egg laying onward (at 25°C). For late larval stages, animals were kept at 25°C. The white prepupa (puparium formation) was used as a developmental reference point for staging pupae.

Hydroxyurea Treatment

Hydroxyurea treatment was made according to de Belle and Heisenberg (1994). Briefly, 50 mg of HU was added to 1 mL of fresh yeast made semiliquid in a microwave oven. Newly hatched L1 or larvae aged 24, 48, and 72 h after larval hatching (ALH) were rinsed from the food and transferred to a drop of the HU-containing yeast paste in a small petri dish. After feeding for 4 h at 25°C, larvae were rinsed, transferred to fresh food, and kept at room

temperature until adult eclosion. Control larvae were exposed to yeast paste lacking HU.

Histochemistry and Immunocytochemistry

To visualize β -galactosidase, larvae, pupae, and adult heads of the GAL4/UAS-*lacZ* transheterozygotes were dissected in Millonig's buffer, fixed in 1% glutaraldehyde (in Millonig's), washed, and stained for β -galactosidase activity with a solution containing 5–10 mg X-Gal/mL dimethylsulfoxide (Brand and Perrimon, 1993). The brains were then either mounted in Faure's solution or dehydrated, embedded in Epon, and sectioned at 10 μ m. The TAU protein in GAL4/UAS-*tau* transheterozygotes was demonstrated by applying a monoclonal anti-TAU antibody (1:2000; Sigma Chemical Co., St. Louis, MO) to 10- μ m cryosections overnight at 4°C and subsequently staining with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). TAU patterns in the larval CNS were also studied in whole mounts. For these preparations, the dissected CNS was treated with the anti-TAU antibody for 2 days at 4°C (with agitation), and the secondary and horseradish peroxidase-conjugated antibodies (Vectastain ABC) were applied each overnight.

RESULTS

Gross Structural Changes in Adult AL after Early Larval HU Treatment

In a first series of experiments designed to analyze structural defects in the AL, we applied HU to early L1 of the *Canton-S* strain and examined the autofluorescence pattern in serial 4- μ m Epon sections. As shown previously (de Belle and Heisenberg, 1994), the AL of HU-treated males and females were reduced equally, measuring only 55–65 μ m from anterior to posterior, compared to 70–80 μ m in untreated flies. In control flies, many landmark glomeruli (Stocker et al., 1990) were identifiable by autofluorescence. After HU treatment some of the glomeruli could still be recognized, but in general they were smaller and less distinct, and some appeared slightly displaced. For example, glomerulus V, which usually protrudes ventrally into the AL cortex, no longer formed this characteristic bulge. Also, the strong lateral indentation between glomeruli VL₂ and DL₂, a feature attributed to interneuron projections from their cell bodies into the AL neuropil, was missing in HU treated flies [cf. Fig. 6(A,B)]. This is most likely because many of these interneurons had been lost. In summary, early larval HU treatment produced specific structural changes in the AL, suggesting that the

cellular elements of the AL were affected in different ways.

Developmental Effects of Late Larval HU Treatment

A 4-h HU application to the early L1 led to reproducible defects of brain structure and slowed post-embryonic development to some extent, but had no effect on the adult eclosion rate. In contrast, HU applied later during larval life dramatically affected viability. Four-hour HU treatment at 24 h ALH (i.e., around first larval moult) or at 48 h ALH (second larval moult) resulted in considerable larval and pupal lethality and only 5–20% adult eclosion (depending on the strain used). Treatment in the mid-L3 (72 h ALH) no longer affected larval survival, but pupal lethality was still between 5 and 50%. Many of the adult survivors were weak and showed subtle eye, cuticular, or wing defects. The most severe effect of HU corresponded with the peak stages of cell proliferation in the nervous system and elsewhere, confirming that HU is not toxic per se, but affects development by ablating large numbers of dividing cells and their descendents (Truman and Booker, 1986; Broadie and Bate, 1991; Ito and Hotta, 1992).

To study possible effects of HU on antennal sensilla, males of one of the GAL4 lines used, GH298, were treated with HU at the developmental stages mentioned above. In adults, the numbers of trichoid sensilla as a representative of olfactory sensilla were determined in whole mounts of third antennal segments (cf. Stocker et al., 1993). While in controls we counted 148 ± 3.2 trichoid sensilla per antenna, the corresponding figures for HU-treated flies were 158 ± 6.1 (HU at 0 h ALH), 141 ± 3.5 (24 h ALH), 149 ± 7.5 (48 h ALH), and 127 ± 5.7 (72 h ALH) [$n = 4 \pm$ standard error (S.E.)]. Analysis of variance showed that differences were significant [$F(4,15) = 4.54$, $p = 0.0134$] and a Student–Newman–Keuls test ($p \leq 0.05$) identified the source of these differences between groups receiving HU treatment at 0 and 72 h ALH only. Basiconic and coeloconic sensilla also appeared to be unaffected by HU treatment up to 48 h ALH (data not shown).

Labeling of *Loel* in GAL4 Line GH298

lacZ and *tau* reporter gene activity labeled a subset of large cells in the AL of GAL4 enhancer trap line GH298 (a P element insertion at 99F). However, complete neuronal morphology was only revealed by the TAU pattern. As estimated from serial cryo-

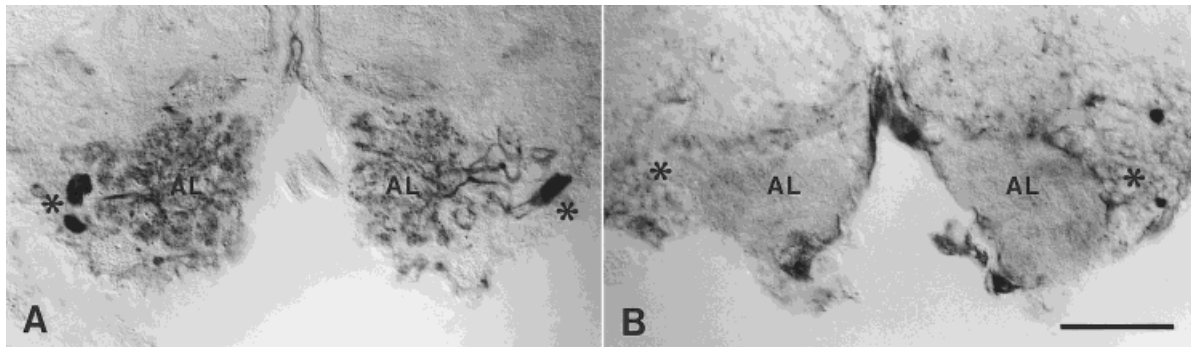


Figure 1 TAU expression in the AL of P[GAL4] line GH298. (A) In controls, the label is localized in local interneurons (LocI), characterized by lateral cell bodies (asterisks) and by an arborization in the entire AL neuropil. (B) After HU application in the early L1, LocI are lost (asterisks). Transverse cryosections; bar = 50 μ m.

sections, we observed about 20 neurons with arborizations in the entire AL neuropil, most of which had their cell bodies lateral to the AL [Fig. 1(A); see also Fig. 6(A)], except two or three that had ventral cell bodies. This pattern fits exactly the shape of LocI (Stocker et al., 1990). Minor expression was also seen in undefined elements having ventral cell bodies and arborizations in glomerulus V, as well as in five to 10 neurons with lateral cell bodies and terminals in glomerulus VL₁ and in the posterior half of the AL (data not shown). The latter elements represent a type of RI, since they project to the LPR. Outside the AL, a subset of other neurons was labeled: in particular, in the suboesophageal ganglion. We found no obvious sexual dimorphism in the expression pattern of line GH298. LocI are also labeled by GAL4 line c739 (Armstrong et al., submitted), but judging from their more compact arborization pattern and dorsolateral location of many cell bodies, these elements may represent a different subtype of LocI.

In line GH298, there was no TAU expression in the AL of L3 or in early pupae, although a subset of other neurons was labeled. From 24 h after puparium formation (APF) onward, *lacZ* expression was seen in a few nuclei ventral to the AL, which may correspond to the two or three LocI having ventral cell bodies in the adult. At 48 h APF, we started to see weak *lacZ* staining in six to 10 cell bodies lateral to the AL, as well as in the RI type mentioned above. In newly eclosed adults, LocI showed the mature *lacZ* pattern, whereas TAU staining was fully established only on the third day. In contrast, on the 10th adult day, *lacZ* expression in LocI faded, while the TAU pattern was still very strong.

Ablation of LocI by Larval HU Treatment

To analyze whether changes in the AL after early larval HU treatment were due to a loss of LocI, we

applied HU to early L1 of line GH298. In adult flies, the AL volume was significantly reduced, confirming the wild-type data (see above) (de Belle and Heisenberg, 1994). Moreover, the TAU pattern within the AL changed dramatically. In fact, the LocI normally stained in this line disappeared entirely, leaving no traces of either their cell bodies or arborizations [Fig. 1(B); see also Fig. 6(B)]. In contrast, none of the other elements expressing GAL4 in the AL were affected (data not shown). Similar to line GH298, HU ablation in line c739 led to the loss of the LocI labeled in this strain (Armstrong et al., submitted). In summary, early larval HU treatment in lines GH298 and c739 has shown clearly that LocI derive from the LNb and suggests that they are born during early postembryonic life (see Fig. 5).

After late larval HU treatment, the adult survivors were fixed immediately after eclosion due to their frequent weakness (see above). In these experiments, we studied mostly *lacZ* patterns, because TAU expression in LocI is delayed (see above). After HU application at 24 h ALH, no LocI were observed in adult flies. In eight of 20 flies treated with HU at 48 h ALH, neurons with lateral cell bodies and arborizations in the entire AL appeared, most likely LocI. After feeding HU at 72 h ALH, LocI patterns were similar to those seen in controls.

Labeling of RI in GAL4 Line GH146

GAL4 line GH146 (a P element insertion at 51B-C) labeled specific elements corresponding exactly to monoglomerular RI, the links between individual glomeruli and the calyx/LPR region. Expression was seen in three clusters of cell bodies: anterodorsal, lateral, and ventral to the AL (termed RIa, RIi, and RIv) [Fig. 2(A,B); also see Fig. 6(A)]. From

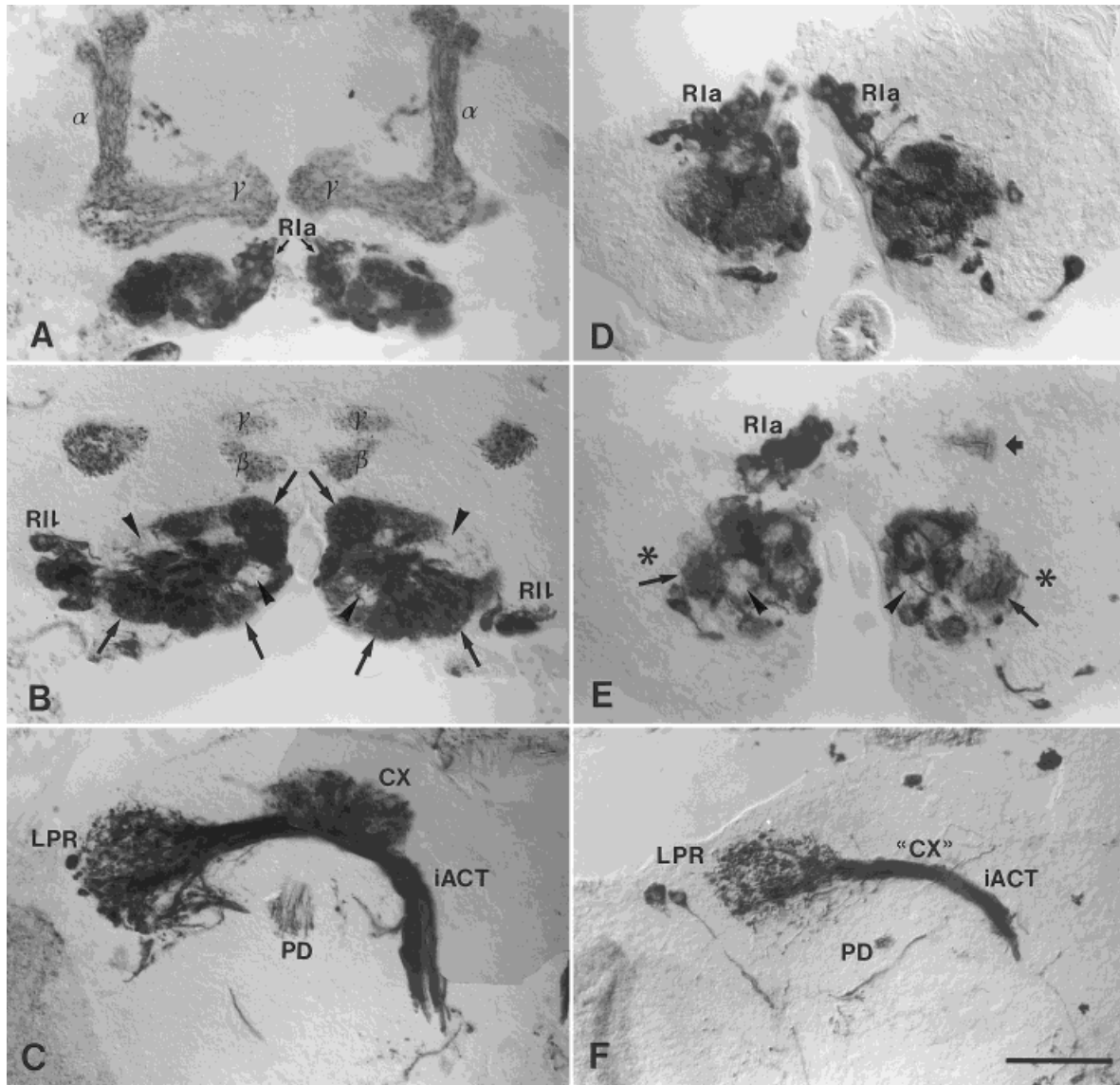


Figure 2 TAU expression in GAL4 line GH146. (A–C) In controls, the label resides in relay interneurons, RIa and RII, whose cell bodies are situated anterodorsal and lateral to the AL, respectively. TAU staining is present in most of the glomeruli (arrows), but not in all of them (arrowheads). RI connect individual glomeruli via the iACT with the calyx (CX) and the LPR. TAU is expressed also in a subset of KC fibers in the α , β , and γ lobes (α , β , γ) and in the pedunculus (PD). (D–F) After HU treatment in the early L1, RIa persist, whereas RII are lost (asterisks). TAU-positive glomeruli (arrows) and TAU-negative glomeruli (arrowheads) are manifest. Axons from the RIa extend to the LPR but do not form collaterals in the presumed calyx region (“CX”). Although no MBs proper are visible, fibers occupy positions reminiscent of KCs, e.g., in the PD and presumably in the γ lobe (thick arrow). The neurons lateral to the LPR (F) are not part of the antennal system. Transverse sections; bar 50 = μm .

cryosections we estimated about 50 RIa, about 40 RII, and 10–15 RIV. The cell bodies of RII overlapped spatially to some extent with those of LocI, although they were generally closer to the AL. TAU staining reflected the glomerular architecture of the periphery of the AL and the fibrous structure of its center [Fig. 2(B)]. This pattern was caused by RI

processes extending from their cell bodies to their glomerular arborizations, and by their output projections (see below). While most glomeruli were heavily stained, a distinct subset of them appeared strikingly empty [Fig. 2(B)]. The majority of labeled RI processes left the AL via the inner antennocerebral tract [(iACT), also called the antenno-

glomerular tract (AGT)] (Stocker et al., 1990). They formed tightly clustered dorsal collaterals in the calyx and established profuse terminal arborizations in the LPR [Fig. 2(C); also see Fig. 6(A)]. Few RI fibers projecting from lateral glomeruli used the middle branch of the ACT (mACT) (Stocker et al., 1990). This tract branches off from the iACT shortly behind the AL, passes below the pedunculus, and extends directly to the LPR [see Fig. 6(A)].

As a novel element, line GH146 revealed a small group of clustered cell bodies ventral to the LPR [see Fig. 6(A)]. Their processes extended directly toward and converged with the iACT near the calyx. They apparently descended in the iACT, but the presence of ascending RI fibers prevented us from identifying whether collaterals were formed in the calyx and if the processes ended in the AL. Of the few additional neurons labeled by line GH146, we noticed a subset of Kenyon cell (KC) fibers in the MB. They extended through the calyx behind the RI collaterals, further through the pedunculus [Fig. 2(C)], and terminated in α , β , and γ lobes [Fig. 2(A,B)]. They did not seem to be associated with particular compartments of the pedunculus and did not correspond entirely with any of the MB GAL4 patterns described before (cf. Yang et al., 1995). None of the expression patterns of line GH146 were obviously sexually dimorphic.

Ablation of a Subset of RI by Early Larval HU Treatment

To study whether the early dividing LNb also gives rise to RI, we fed HU to early L1 of line GH146. This treatment appeared to eliminate MB calyces, pedunculi and α , β , and γ lobes in the adult, in agreement with data in the wild-type and other GAL4 lines (de Belle and Heisenberg, 1994; Armstrong et al., submitted). In the AL, RIa and RIv remained unaltered, still consisting of about 50 and 10 cells, respectively [Fig. 2(D); also see Fig. 6(B)]. In contrast, HU treatment severely reduced RII to not more than one or two neurons, and sometimes none [Fig. 2(E); see also Fig. 6(B)]. Yet, despite the lack of these neurons and perhaps all of the LocI (see above), the glomerular appearance of the AL persisted (as revealed by the expression pattern of the remaining RI) [Fig. 2(E)]. Moreover, GAL4-positive and GAL4-negative glomeruli were still manifest. All of the surviving RI fibers converged toward the iACT and segregated immediately behind the AL into a dominant iACT fraction and a smaller mACT fraction. The most prominent change in iACT fibers was the lack of collaterals in the presumed calyx region [Fig. 2(F); see also Fig.

6(B)]. Yet, these axons extended further laterally and terminated in the LPR, exactly as seen in control flies. The reduced number of RI fibers was reflected by a smaller iACT diameter and a lower total density of LPR arborizations [Fig. 2(F)]. The mACT did not seem to be affected by HU treatment, suggesting that all mACT fibers have anterodorsal cell bodies [see Fig. 6(B)].

Other elements that persisted after HU treatment were the presumed descending fibers in the iACT [see above, and Fig. 6(B)]. However, if they normally arborize in the calyx, these connections must have been lost. Moreover, a small number of GAL4-expressing fibers clustered in a region corresponding to the normal calyx. From there they extended along a pathway reminiscent of the pedunculus and presumably the γ lobe, similar to a subset of KC fibers seen in untreated flies [Fig. 2(E,F); see also Fig. 6(B)]. No fibers were observed in a tract comparable to the α -lobe.

In summary, early larval HU treatment led to the loss of RII but spared RIa and RIv. This suggests that RII derive from the LNb and are born early in postembryogenesis, whereas RIa and RIv have their terminal divisions later in larval life (see Fig. 5). Early HU treatment also has severe effects on the MBs (without resulting in their complete loss), but appears to leave the LPR region intact. Consequently, changes in the remaining RI are restricted to the lack of collaterals in the calyx neuropil, whereas LPR arborizations seem to be unaffected.

Changes in RI after Late Larval HU Treatment

When HU was applied for 4 h at 24 h ALH to line GH146, the AL of the few adults recovered was extremely reduced in size, comprising only 20–30 μm in the longitudinal axis (compared to 55–65 μm after HU treatment in the early L1). Very few elements were labeled in the AL, most of which were of the RII or RIv type, exceptionally of the RIa type [Fig. 3(A)]. A limited number of fibers comprising two or three bundles extended along the iACT and terminated in the LPR [Fig. 3(B)]. In contrast to early HU ablation, MBs were manifest, although much smaller than in controls. Accordingly, iACT fibers showed limited collateral formation in the calyx region.

After feeding HU at 48 h ALH, representatives of each of the three RI clusters were observed, composed of an estimated 20–30 RIa, 10–20 RII, and five to 10 RIv [Fig. 3(C)]. The distribution of RI processes in the AL appeared to be more homogeneous than in the controls, although the glomerular

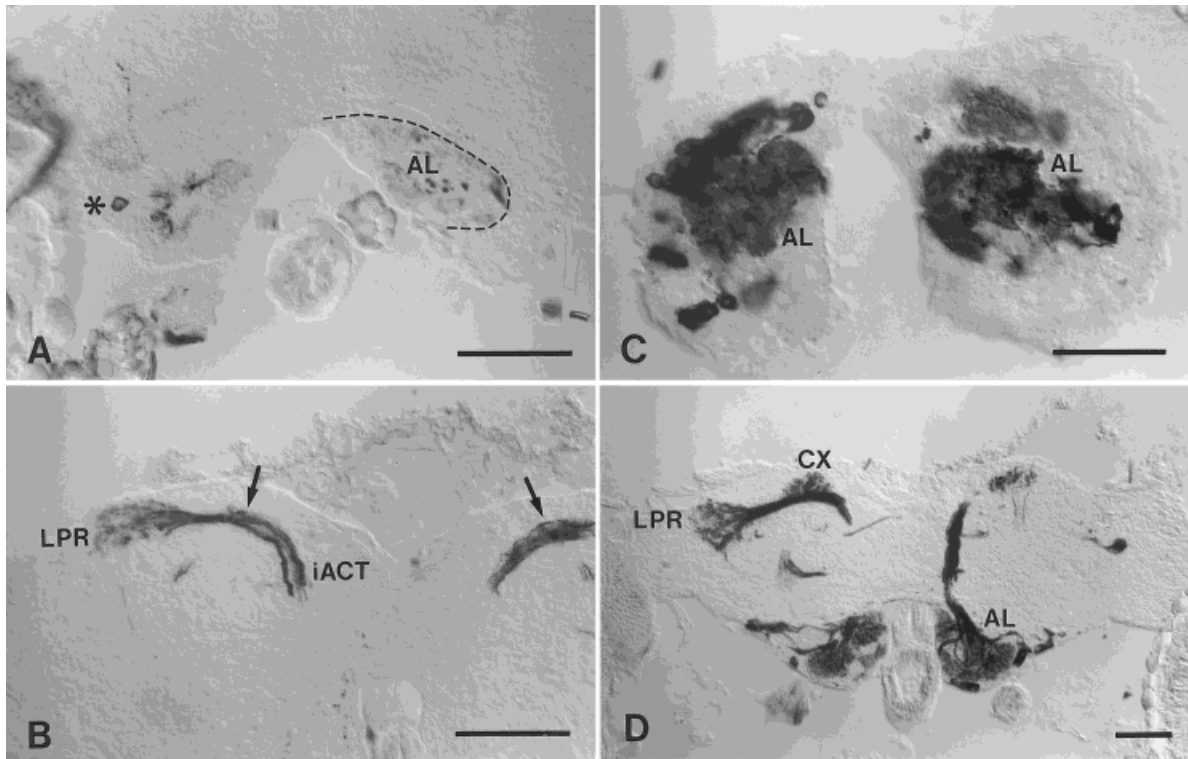


Figure 3 (A,B) TAU expression in line GH146 following HU treatment 24 h after larval hatching (ALH). Only few arborizations are visible in the severely reduced AL (outlined). Most of the labeled RI have lateral cell bodies (asterisk). Two to three bundles of fibers projecting along the iACT to the LPR show limited collateral formation in the calyx region (arrows). (C) After HU application at 48 h ALH, arborizations in the AL appear to be more homogeneous than in controls. (D) HU treatment 72 h ALH leads to similar TAU patterns in the AL, calyx, and LPR as in controls. (A–C) Transverse sections; (D) oblique section; bar = 50 μ m.

character was still evident. The morphology of all other pattern elements labeled by line GH146 appeared to be normal. Yet, MBs and the RI arborizations in the calyx were both still reduced in size. After HU treatment at 72 h ALH, the TAU patterns in the AL, calyx, and LPR were similar to those observed in the controls [Fig. 3(D)].

Expression in RI Begins at the Second Larval Molt

We found no TAU staining in the brain of line GH146 at 24 h ALH. However, at 48 h ALH, six to 10 neurons with arborizations in the larval AL (Stocker et al., 1995) and axons extending into a region corresponding to the adult calyx became visible [Fig. 4(A)]. In mature L3, the number of these neurons had increased to about 20. All of them had lateral cell bodies and branched in the entire larval AL, which lacked an obvious glomerular structure [Fig. 4(B)] (Stocker et al., 1995). Their processes

exhibited all of the features characteristic of adult RI. They followed a pathway similar to the iACT and established two terminal fields of arborization: a compact one in the calyx region, and a less dense one in the larval LPR [Fig. 4(C)].

In young pupae (24 h APF), many more neurons were labeled in the AL region. Their cell bodies belonged to one of two partially overlapping clusters, lateral and anterodorsal to the AL, and their numbers were roughly the same as those in the RI_l and RI_a adult clusters. The anterodorsal cell bodies had no detectable outgrowths [Fig. 4(D)], whereas the lateral cell bodies sent processes into the AL neuropil [Fig. 4(E)]. The arborization in the AL still lacked an obvious glomerular pattern. Compared to the L3, the numbers of processes projecting to calyx and LPR had significantly increased [Fig. 4(E)]. Most of them belonged to RI, but four or five fibers corresponded clearly to the putative descending elements present in the adult (see above). Neither mACT fibers nor KCs were labeled. At 48

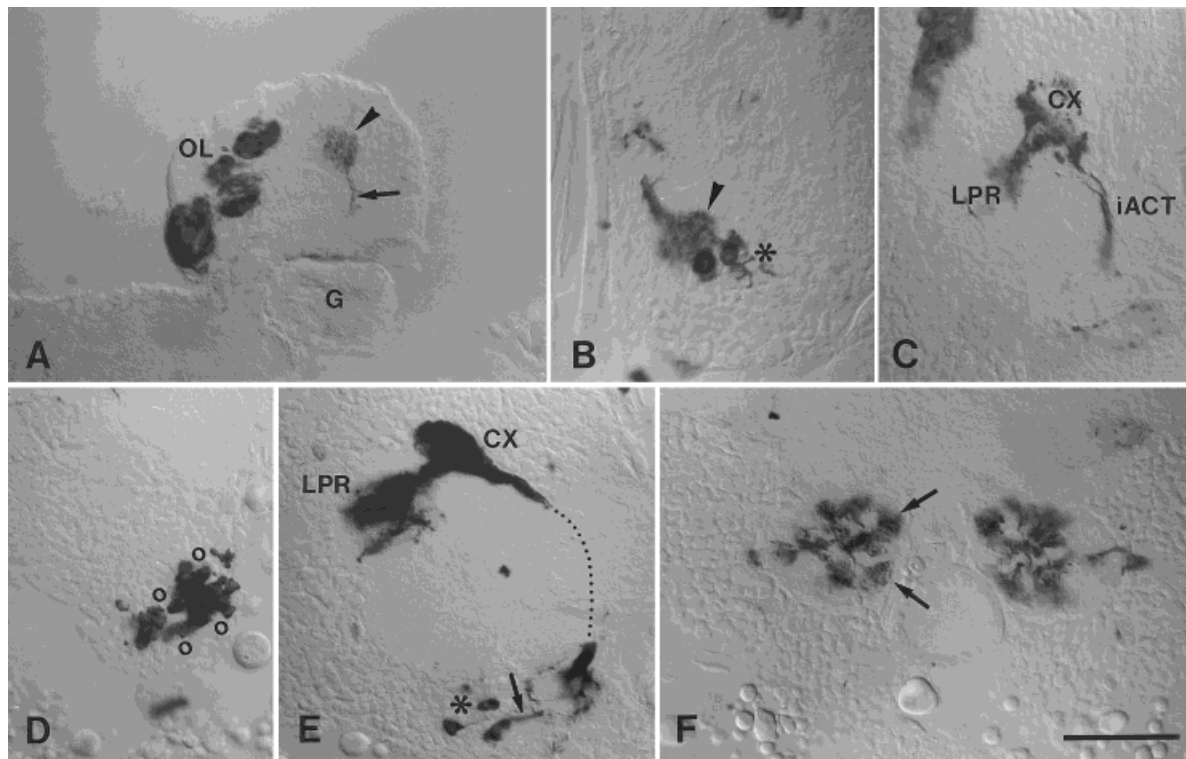


Figure 4 Developmental TAU expression in RI as shown by line GH146. (A) At 48 h ALH, RI-like neurons extending via a pathway similar to the iACT (arrow) establish arborizations in the presumed calyx region (arrowhead). Strong expression is also present in the optic lobes (OL). G gut. (B,C) At 72 h ALH, well-identifiable RI-like neurons with lateral cell bodies (asterisk) connect the nonglomerular larval AL (arrowhead) via the iACT to the CX and LPR. (D,E) At 24 h after puparium formation (APF), TAU-labeled anterodorsal cell bodies (open circles) have not yet formed outgrowths, whereas the mature RI are characterized by lateral cell bodies (asterisk) and processes (arrow). Their projections along the iACT (stippled) to the CX and LPR are similar to the adult. (F) At 48 h APF, AL arborizations are becoming glomerular (arrows). (A) Sagittal section; (B,C) horizontal sections; (D–F) transverse sections; bar = 50 μ m.

h APF, RIa had also formed processes. The pattern of RI arborization revealed a glomerular structure in the AL [Fig. 4(F)], and KCs had begun expressing GAL4.

DISCUSSION

Neuroblast Ablation

Evidence presented here and elsewhere (Timson, 1975; Truman and Booker, 1986) favors HU as a potent ablating agent of dividing cells without being otherwise toxic. Feeding HU for 4 h in early L1 does not affect viability (except for slowing postembryonic development), but leads to the loss of most MB neuropil and to an AL volume reduction (de Belle and Heisenberg, 1994). Furthermore, HU treatment in the mid-L3 does not result in any sig-

nificant changes in brain structure. In contrast, HU applied at 24 or 48 h ALH reduces adult survivorship by 80–95%. This corresponds to periods during which the entire set of neuroblasts has begun to proliferate (Ito and Hotta, 1992; Stocker et al., 1995) and mitotic activity elsewhere may be at its peak as well (Truman and Booker, 1986). HU applied at this time certainly leads to the highest reduction of cellular offspring possible (Fig. 5). Before that period, most of the neuroblasts are spared because they have not yet entered the mitotic stage, and after that period most of the progeny have already been born.

We cannot exclude the possibility that individual mitotic cells may have been affected differently by HU, because they may have been exposed to somewhat different concentrations. Yet we consider this unlikely, since HU was fed for 4 h at a high concen-

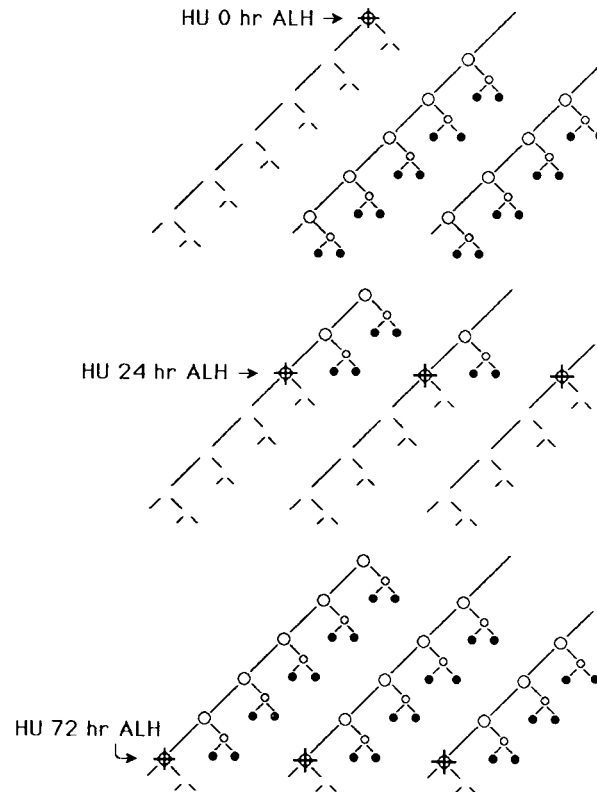
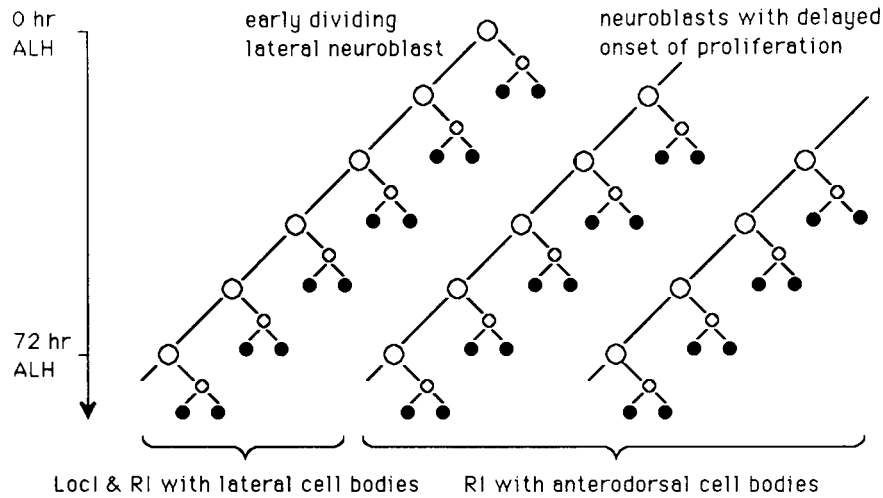


Figure 5 (Upper) Putative cell lineage of AL interneurons. Large and small open circles refer to dividing neuroblasts and ganglion mother cells, respectively. Filled dots indicate postmitotic cells, e.g., interneurons. The time scale after larval hatching (ALH) is shown on the left. (Lower) Effects of HU treatment at various times ALH. Neuroblast ablation is shown by crossed circles, the resulting lack of interneurons by the absence of dots. The highest cell loss obviously results from HU application at an intermediate stage.

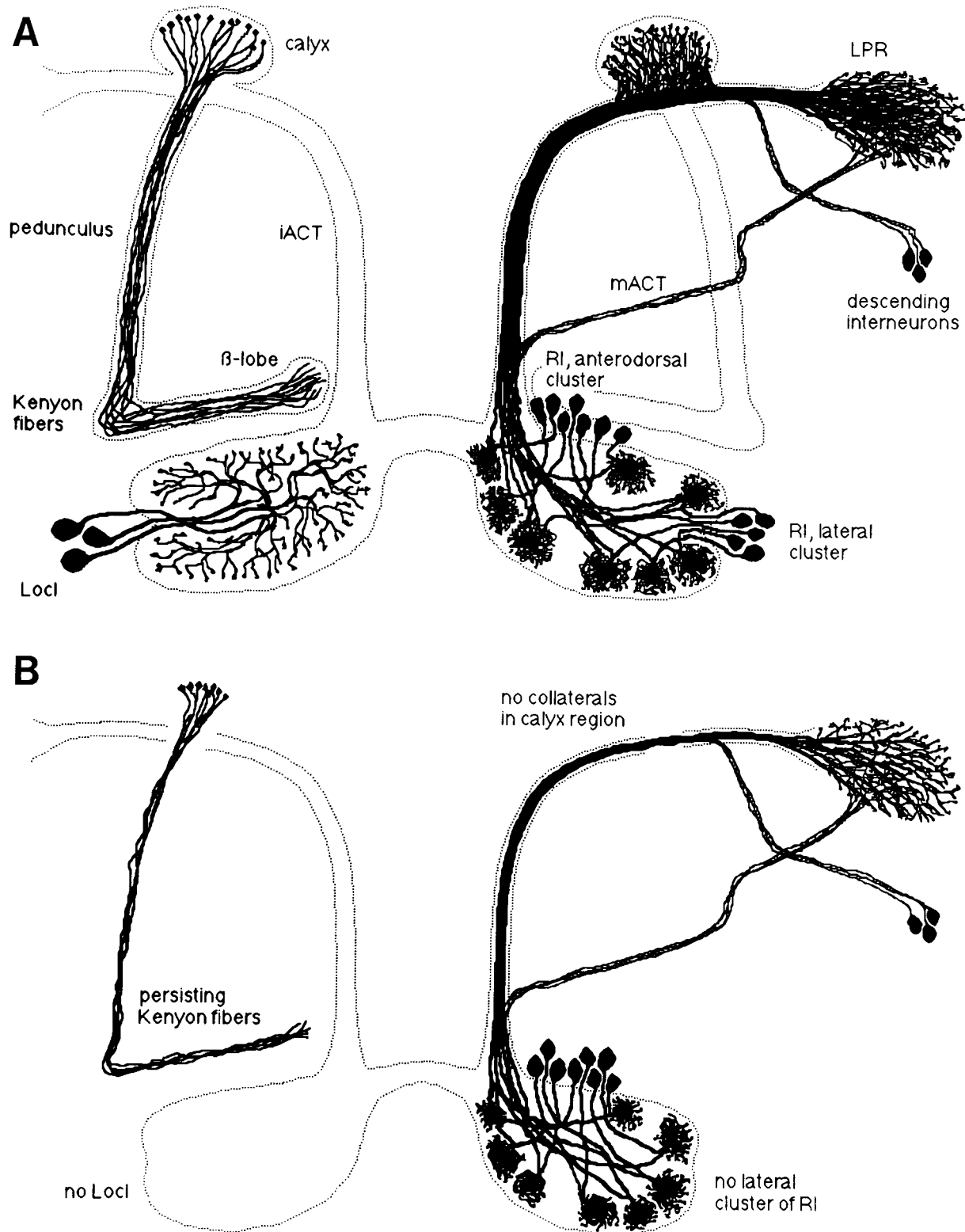


Figure 6 (A) Summary diagram of AL and MB expression patterns of the two GAL4 lines used. Left and right sides show different elements, separated for clarity. (B) Major pattern changes after HU treatment in the early L1. iACT, mACT = inner and middle antennocerebral tract, respectively. Transverse views.

tration during actively feeding stages (J.S. de Belle, unpublished data). This should have allowed for sufficient exposure in every dividing cell (Broadie and Bate, 1991). In agreement with this assumption, we observed little variation in the numbers of surviving labeled cells in each series of experiments. Furthermore, low variability has been reported in other HU ablation studies using the same protocol (de Belle and Heisenberg, 1994; J.S. de Belle, unpublished data).

GAL4 Expression Patterns in Lines GH298 and GH146

Because of the current lack of data on cell numbers in the AL, we cannot determine if the ± 100 RI labeled by line GH146 and the ± 20 LocI shown by GH298 represent the entire set of these interneurons. An estimate of RI numbers is given by the number of fiber profiles in the iACT. At its exit from the AL, the iACT is composed of about 200 fibers, but many of them appear to belong to other types of neurons (Stocker et al., 1990). In *Manduca* with an AL composed of 64 glomeruli (Rospars and Hildebrand, 1992), 360 LocI and 360 monoglomerular RI have been described (Homberg et al., 1989). *Periplaneta* with about 125 glomeruli was reported to have < 250 RI but three times as many LocI (Boeckh and Ernst, 1983). Extrapolation from these data leads to 70–200 LocI and 100–150 RI in *D. melanogaster*, corresponding to its approximately 35 glomeruli (Stocker et al., 1990). However, this does not take into account the smaller AL size compared to these other species. In fact, mass Golgi impregnations rarely showed more than one RI per glomerulus (Stocker et al., 1990). This suggests that the numbers of RI labeled by line GH146 may be not too far from the true numbers, although the lack of staining in certain glomeruli indicates a lack of expression in a subset of RI. In contrast, the numbers of LocI in line GH298 may be largely underrepresented.

The temporal control that enhancers exert over gene expression is reflected in a dynamic expression of reporter genes during development, or during adult life (Helfand et al., 1995). GAL4 lines GH146 and GH298 demonstrate two extremes of such patterns. In line GH146, expression begins when the RI extend their processes, as shown by the RIa cluster at 24 h APF. Since expression persists in the adult, the underlying gene may function both during development and in adulthood. In contrast, line GH298 shows no larval or early pupal expression in LocI, although early and late larval HU treatments

demonstrated that they descend from the early dividing LNb, and that some of them are present already at the second larval moult. Obviously, GAL4 is expressed in LocI only at (or shortly before) eclosion, suggesting a purely adult role of the putative gene reflected by this pattern. Interestingly, in GH298 adults, the temporal expression patterns of the two reporter genes used were different. After eclosion, LocI showed the mature *lacZ* pattern, whereas TAU staining was fully established only 2 days later. In contrast, on the 10th day *lacZ* expression faded, while the TAU pattern was still very strong. As suggested for developing antennal afferents (Tissot et al., 1997), the *lacZ* reporter gene may monitor GAL4 expression more precisely than the *tau* gene, whose expression appears to be delayed for hours or even days.

Lineage and Birth Date of Olfactory Interneurons

This study demonstrates that LocI and RIi are derived from the early dividing LNb, whereas RIa, RIv, and additional types of AL interneurons are formed by other neuroblasts that begin to divide later during larval life (Fig. 5). Obviously, there is a correlation between the position of the LNb and the cell body locations of its offspring, both of which are lateral to the AL. In contrast, no comparable relationship exists for RIa and RIv. The approximately 12 pairs of neuroblasts dividing in the vicinity of the AL in the late L3 are uniformly distributed and show no anterodorsal or ventral clustering (Stocker et al., 1995). Hence, in the absence of specific neuroblast markers, the cell lineage of these interneurons remains unknown.

Retention of Larval RI through Metamorphosis

There are some reports in *Manduca* on the metamorphic fate of AL interneurons. Presumed γ -aminobutyric acid-ergic LocI with lateral cell bodies occur both in the larval and adult AL, but since immunoreactivity ceases for some time after pupation, it is not certain whether these neurons are the same (Homberg and Hildebrand, 1994). Odor-responsive larval RI projecting to the LPR were described, but their fate remains unknown (Itagaki and Hildebrand, 1990). The differentiation of RI was studied only from the early pupal stage onward (Malun et al., 1994). The only documented case of persistence through the larval–pupal transition refers to a sero-

tonergic interneuron that arborizes in the entire AL and extends processes to the ipsilateral and contralateral LPR (Kent et al., 1987; Oland et al., 1995). Except for some restructuring in the AL, this neuron maintains the same basic morphology in the adult as in the larva.

As shown by the developmental expression of line GH146, some of the RII appear as early as the second larval moult and increase gradually in number until the end of larval life. This is consistent with data obtained from HU application at 24 h ALH which indicate that some RII are in fact born even before the first larval moult. As judged from their fully developed architecture, larval RI are very likely active, at least in L3. The RI labeled by GH146 can be followed continuously during metamorphosis, and their projection pattern to higher brain centers remains essentially unchanged. We did not examine the iACT during metamorphosis for evidence of larval RI fiber retraction and replacement by adult RI fibers in a manner similar to that described for MB KCs (Technau and Heisenberg, 1982). Thus, the possibility that this occurs cannot be completely ruled out. However, large-scale retraction of RI processes seems rather unlikely, because no degenerating fiber profiles were reported in the iACT during metamorphosis (Technau and Heisenberg, 1982). Hence, these data suggest that larval AL interneurons may persist through metamorphosis in *Drosophila*. Moreover, they indicate for the first time in insects the retention of some of the uniglomerular RI. These neurons link the AL to the MB, two brain centers that have been implicated in olfactory learning (Heisenberg et al., 1985; Nighorn et al., 1991; de Belle and Heisenberg, 1994; de Belle, 1995; Hammer and Menzel, 1995). Recent reports suggest that different forms of olfactory memory may be retained in *Drosophila* during metamorphosis (Tully et al., 1994; A.K. Guo and K.G. Götz, unpublished data). If metamorphic long-term memory in fact exists, identification of the underlying neural elements or circuits would be crucial. RI with a putative larval and adult function may be possible candidates involved in such a task.

The presence of many LocI in line GH298 after HU ablation as early as 48 h ALH indicates that some (or all) of these neurons have been born during larval life (see above). Since they do not express GAL4 until adult eclosion, it remains an open question whether they exhibit in the larva the features characteristic of LocI. Alternatively, they may correspond to neurons in *Manduca* which are born during larval life but remain blocked in an immature condition until late pupal stages (Truman, 1990).

Metamorphic Changes in the AL and in Higher Olfactory Centers

Previous observations of the first appearance of glomeruli 48 h APF (Stocker et al., 1995) are confirmed here by the developmental pattern of line GH146. The transformation from a nonglomerular larval AL to a glomerular adult AL (Tissot et al., 1997) must be accompanied by changes in the dendritic arborization of persisting RI. The simultaneous labeling of many RI in mature L3 prevented us from identifying the dendritic pattern of single neurons. However, the lack of a glomerular pattern argues for a homogeneous arborization of individual RI fibers in the entire larval AL.

In *Manduca* it was shown that the differentiation of antennal glomeruli requires both afferent input and the presence of glial cells (Oland and Tolbert, 1996). The present study allows one to examine possible effects of a third parameter: the number of target interneurons. As our calculation of antennal sensilla suggests, HU application does not affect afferents up to 48 h ALH, consistent with reported antennal cell proliferation in the late L3 and early pupa (Postlethwait and Schneiderman, 1971; Ray and Rodrigues, 1995). Also, glial proliferation in the AL peaks only after puparium formation (Stocker et al., 1995). While the lack of most (perhaps all) of the LocI and of RII by early larval HU treatment did not abolish glomeruli, ablation of additional elements by later HU treatment led to a more homogeneous distribution of the arborizations of surviving RI, but still did not eliminate the glomerular architecture completely. Thus, even a significant loss of target interneurons does not abolish glomeruli formation. Experiments are under way to analyze the fate of individual glomeruli in more detail.

Early larval HU ablation was shown to spare a fiber bundle in the MB, which was interpreted to consist of either KCs of embryonic origin or of extrinsic elements (de Belle and Heisenberg, 1994). Line GH146 labels a small subset of KC fibers which survives after early larval HU treatment, suggesting that the first explanation is the correct one.

In the MB, extensive metamorphic reorganization has been reported involving breakdown and regrowth of larval KC fibers and the addition of new, adult-specific KCs (Technau and Heisenberg, 1982). Similar to the AL, rearrangement is very likely accompanied by connectivity changes in the major inputs to the KCs, the collaterals formed by RI in the calyx. Neither is it known how this rewiring is controlled nor how the presynaptic and postsynaptic elements interact during the initial estab-

ishment of connections. Our data from line GH146 show that MB ablation leads to the complete lack of RI collaterals in the calyx [Fig. 6(B)], most likely because their normal targets, the KCs, are missing. Other structural characteristics of RI remain unchanged: in particular, the terminal arborizations in the LPR. Thus, RI survival does not depend on the establishment of synaptic connections with KCs, probably because RI possess secondary targets in the LPR. Moreover, the lack of calyx collaterals despite persisting embryonic KC fibers suggests that these are not the targets of RIa and RIv, although the presence of minor connections cannot be entirely ruled out. Hence, embryonic and postembryonic KCs may possibly serve different functions in the adult.

Hydroxyurea ablation of LocI and RII has a striking effect on olfaction in *Drosophila* (de Belle and Heisenberg, 1994; J.S. de Belle, unpublished data). Varying degrees of anosmia to a wide range of odors can now be attributed to specific connectivity deficits, either among AL glomeruli or between the AL and higher olfactory centers in the protocerebrum. Construction of an olfactory pathway functional map is currently in progress.

Our data show that P[GAL4] strains in combination with HU ablation have opened a new access to the developmental and functional analysis of the olfactory system. The GAL4 lines presented here provide the first tools available for studying specific types of AL interneurons in *Drosophila*. Besides their usefulness as cellular markers, they offer all the experimental potential of enhancer trap technology (Brand and Dormand, 1995). Future genetic and molecular studies of the underlying genes will provide us with a better understanding of developmental interactions and functional properties of the elements of the olfactory system.

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