A central neural circuit for experience-independent olfactory and courtship behavior in *Drosophila melanogaster*

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We have studied the function of the major central olfactory pathway in fruit flies. Key elements of this pathway, the projection neurons (PNs), connect the antennal lobes with the lateral protocerebrum both directly and indirectly, the latter via the mushroom bodies (MBs). Transgenic expression of tetanus toxin in the majority of PNs and few MB neurons leads to defects in odor detection and male courtship. Considering behavioral data from flies lacking MBs, our results argue that the direct PN-to-lateral protocerebrum pathway is necessary and sufficient to process these experience-independent behaviors. Moreover, the involvement of an olfactory pathway in male courtship suggests a role of volatile attractive female pheromones in *Drosophila*.

Providing structure–function relationships is a central issue in behavioral neuroscience (1, 2). The development of the enhancer trap technique in *Drosophila* (3, 4) made it possible to manipulate subsets of identified neurons in a noninvasive manner. In particular, the P[GAL4]/UAS system allows ectopic expression of any gene of interest (4). When expressing tetanus toxin light chain (TeTxLC) (5), synaptic transmission is locally blocked, and the behavioral consequences can be observed (6–9).

We investigate the function of projection neurons (PNs), the major elements of the central olfactory pathway of *Drosophila melanogaster* (Fig. 6) (10). Olfactory receptor neurons project to the antennal lobes (ALs), the first-order olfactory brain area. The information is then transmitted by PNs via the antennocerebral tract to two target areas, the mushroom bodies (MBs), involved in experience-dependent olfactory processing (2, 11), and the lateral protocerebrum (LPR) (12). Because subsets of MB extrinsic neurons project to the LPR (13, 14), the PNs provide the LPR with olfactory information both directly and, indirectly, via the MBs.

Flies with an intact direct pathway, lacking the indirect one, are impaired in experience-dependent but not -independent olfactory behavior (15). We have generated a P[GAL4] line, GH146, which enables us to shut down about two-thirds of all PNs (Figs. 1 and 6) (16) through ectopic expression of TeTxLC. Both the direct and the indirect input into the LPR are thus blocked, which allows us to investigate possible effects on experience-independent olfactory processing. We show that odor detection and male courtship are severely impaired. We also test gustatory processing to address the question of effect specificity. Furthermore, neurons showing reporter gene expression in the visual system are identified as necessary for movement detection.

Materials and Methods

***Drosophila* Strains.** P[GAL4] line GH146 was described in ref. 16. UAS-reporter strains were UAS-*lacZ* (4), UAS-*tau* (17), UAS-*tra* (18), and the UAS-TeTxLC line TNT-E (called TNT for simplicity) (5). These lines and the wild-type strain Canton-S (CS) were used as controls in behavioral tests.

Immunocytochemistry. β-Galactosidase and antibody staining of whole mount preparations and sections was done according to ref. 8. Anti-TAU antibody was from Sigma, and anti-TeTxLC was kindly provided by H. Niemann (Universität Hannover, Hannover, Germany). The Vectastain ABC system was used for detection of primary antibodies (Vector Laboratories).

Olfactory Choice Assay. Olfactory choice assays were carried out in the dark by using a T-maze apparatus (19). About 50 males or 50 females, 4–5 d old, were inserted into the apparatus, which was connected to two constant air streams, one of which carried the test substance. After 90 sec, the flies in both tubes were counted. A response index was calculated as $No - Nc/No + Nc$, No and Nc being the number of animals inside the odor and control tubes, respectively. Odors tested were ethyl acetate (Fluka), trans-2-hexenal (Aldrich), 2-hexanol (Aldrich), and 1-octen-3-ol (Fluka). All substances were diluted in paraffin oil (Fluka). To determine the threshold of response for GH146/TNT heterozygotes, we first tested a range of dilutions. The concentration leading to clearly abnormal behavior was then used for additional tests.

Proboscis Extension Response. Tests were done as described in ref. 20. Single 3-d-old females and males were starved for 24 h, anesthetized with CO_2, then fixed on slides with tape and modeling clay and left for 2 h in a humid box to recover. Proboscis extension was elicited by touching the tarsi of the forelegs with a drop of sucrose solution. Each fly was tested five times by using the same sucrose concentration. To avoid habituation, we waited at least 30 sec between tests.

Visually Induced Head Roll. Rotation of the visual field around a fly leads to syndirectional turning of the head (21). The angle of the head roll response was measured by gluing 2-d-old females to a manipulator and placing them in an arena. The stimulus was a grating of moving stripes (60° pattern wavelength, 1.2 Hz contrast frequency). The flies were videotaped, and the angle between head positions for clockwise and counterclockwise stimulation was measured.

Fixation Behavior. Pattern-induced orientation of walking flies was analyzed in an illuminated arena (29-cm diameter, 64-cm height) (22). Flies with wings cut off were placed in the center of a circular arena and allowed to walk freely. The arena was

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Abbreviations: AL, antennal lobe; CI, courtship index; CS, Canton-S; LPR, lateral protocerebrum; MB, mushroom body; PN, projection neuron; TeTxLC, tetanus toxin light chain.

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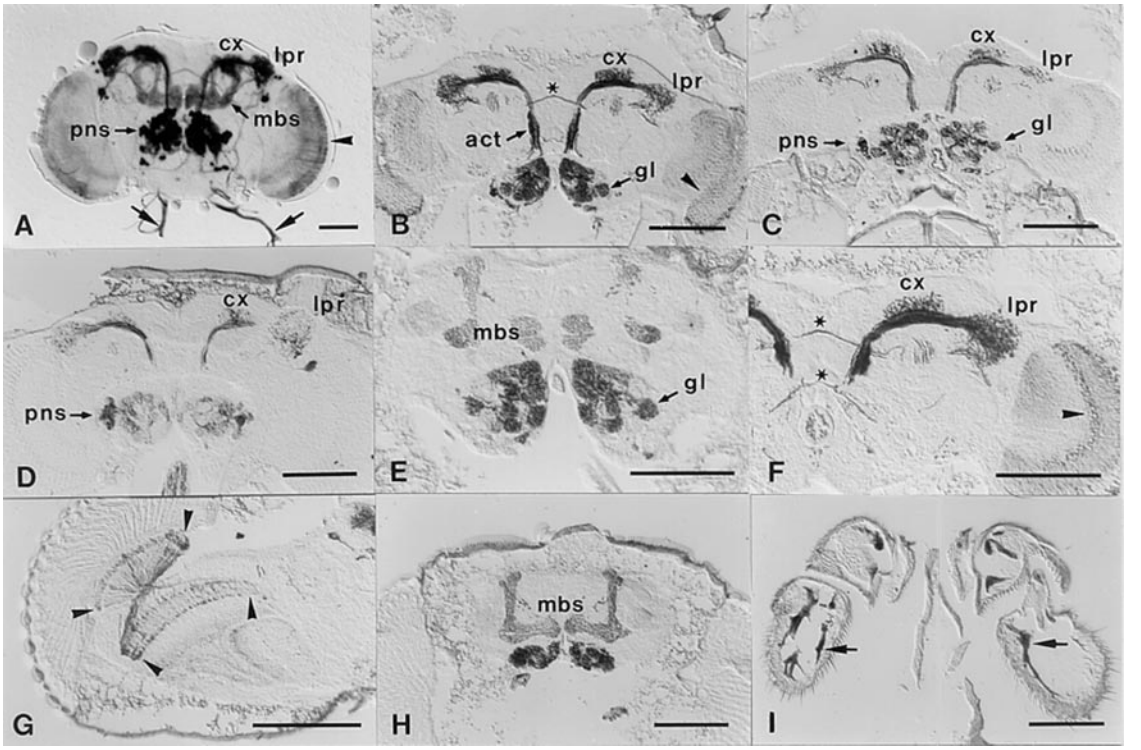

Fig. 1. Adult expression pattern of P[GAL4] line GH146 as visualized by lacZ (*A*), TAU (*B*, *C*, *E–I*), and TeTxLC (*D*) reporters. In *C*, TAU and TeTxLC are coexpressed (TeTxLC pattern not shown). lacZ, TAU, and TeTxLC patterns look similar (cf. *A–D*), although the latter seems weaker, very likely because of the lower affinity of the TNT antiserum. GH146 shows strong expression in projection neurons (pns), which connect antennal lobe glomeruli (gl) via the antennocerebral tract (act) with the mushroom body calyx (cx) and the lateral protocerebrum (lpr). (*E*, *F*) Close-ups of glomerular arborizations, and target regions of PNs, respectively. Additional expression occurs in a few MB interneurons (*A*, *E*, *H*, mbs), in scattered bilateral neurons (*B*, *F*, asterisks), in a subset of lamina monopolar cells (*A*, *B*, *F*, *G*, arrowheads) projecting to three layers of the medulla (*G*, lower arrowheads), and in unidentified neurons arborizing in the lobula and lobula plate (*G*, weak label in neuropil on bottom). All peripheral nerves exhibit glial expression (*A*, arrows) including those within the antenna (*I* , arrows). (*A*) Whole mount, (*B–F*, *H*, *I*) transverse cryosections, (*G*) horizontal cryosection. *B–D* are assembled from two to three sections each. (Bars = 100 μm.)

divided in 12 30° segments. The segment in which flies crossed a measuring circle (19-cm diameter) was recorded. A fixation event was counted when the segment in which they crossed the measuring circle contained a vertical stripe, the position of which was changed halfway during experiments for each animal. The fixation efficiency was then calculated. The stimuli consisted of two opposing stripes of 10° or 60°, respectively.

Courtship Tests. Courtship was tested in small circular Teflon chambers (diameter 11 mm, height 2 mm) (23). Four- to five-day-old males and intact females were introduced without anesthesia by aspiration into the chambers. Courtship behavior of single fly pairs was observed under a binocular microscope for 10 min or until copulation occurred. A courtship index (CI) was calculated as the percentage of time the male spent performing courtship behavior (24). GH146/TNT were tested in white or red light (Kodak Safe-light filter no. 1; light transmission is zero between 220 and 610 nm and >10% between 610 and 900 nm), the latter condition to rule out visual contributions to courtship (23). The tests of GH146/UAS-*tra* were performed under white light only because of the lack of significant effects. To exclude reciprocal courtship, object flies were anesthetized on ice and decapitated (18).

Statistical Analysis. Experiments, which resulted in normally distributed data, were evaluated by using ANOVA. For not normally distributed data, statistical analysis was done by using the nonparametric method of Kruskal-Wallis (25).

Results

Expression Pattern of P[GAL4] Line GH146. The expression pattern of line GH146 as shown by TNT, GFP, TAU, and lacZ reporters (5, 17), was qualitatively similar but varied somewhat in intensity (Fig. 1 *A–D*). To exclude the possibility that TeTxLC expression causes cell death during postembryonic development, we coexpressed TAU and TeTxLC in a GH146-dependent manner. TAU staining in these flies was qualitatively the same as TAU staining in flies lacking TeTxLC expression (Fig. 1 *B* and *C*), suggesting that neurons develop normally even if they express TeTxLC. Unlike larval chemosensory afferents labeled by another P[GAL4] line (8), none of the neurons expressing TeTxLC in GH146 had swollen terminal arbors.

The most intensely stained brain elements of line GH146 are about 100 PNs of the AL (16). Roughly 90 of them establish dendritic arborizations in one of the 43 AL glomeruli (Fig. 1 *A–E*) (26) and send their axon via the antennocerebral tract to the MB calyx and the LPR (Fig. 1 *A–D*, *F*). The remaining 5–10 labeled PNs are mostly of the polyglomerular type, whose fibers bypass the calyx and extend directly to the LPR (27). Three lines of evidence suggest that the labeled PNs represent the majority of this cell type in *Drosophila*: (*i*) the presence of stained arbors in most of the glomeruli, (*ii*) the tight labeling of the antennocerebral tract, and (*iii*) an extrapolation from the known numbers of PNs per glomerulus in other insects (16). Only few additional elements are labeled by GH146 in the adult: (*i*) two to three interneurons with cell bodies near the LPR (Fig. 1 *A* and *D*) and fibers toward the antennocerebral tract, (*ii*) a small set of MB interneurons (Fig. 1 *A*, *E*, and *H*), (*iii*) a subset of lamina monopolar cells projecting to three medulla layers—most likely L1 and L2 (Fig. 1 *A*, *B*, *F*, and *G*)—and of neurons arborizing in one layer of the proximal medulla and lobula, as well as in specific lobula plate layers, (*iv*) a few bilateral neurons in the

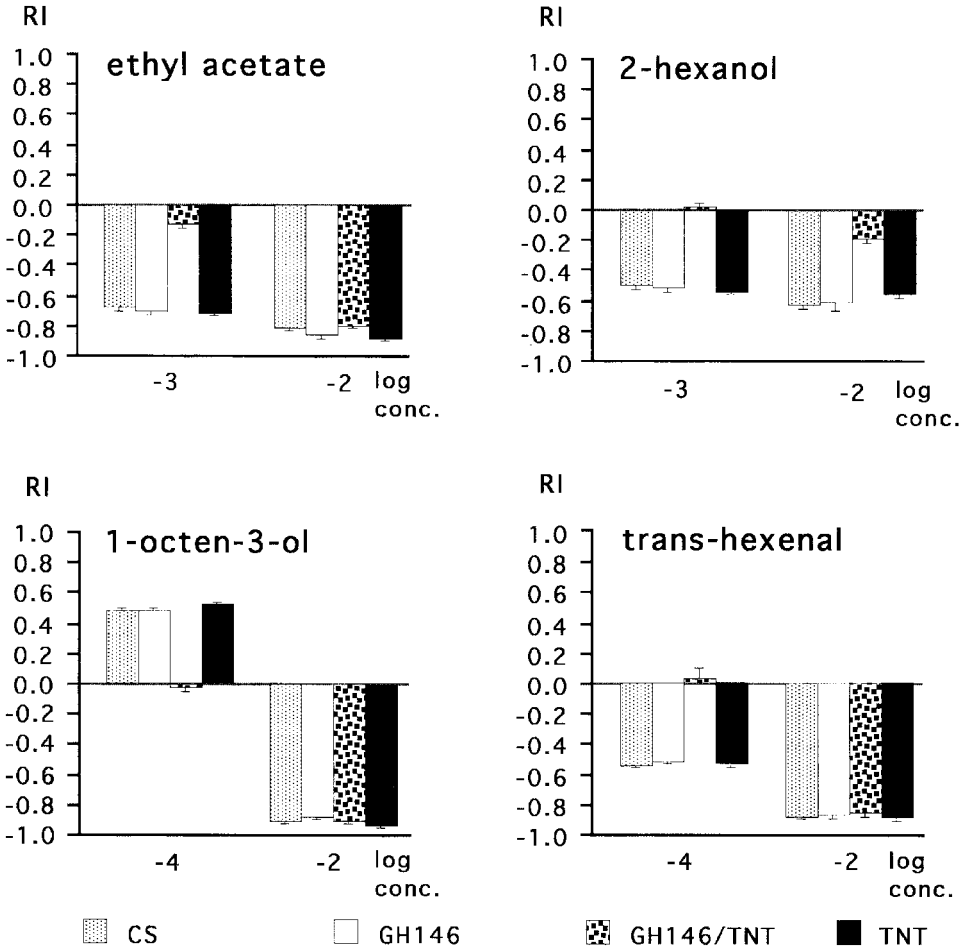

Fig. 2. Olfactory responses in the T-maze assay. For each genotype, 10 independent tests of 50 flies each were carried out. Dilutions are indicated as log units and refer to solutions over which air is blown into the T-maze apparatus. At low concentrations (10^{-3} and 10^{-4}), GH146/TNT flies show no preference (Kruskal–Wallis; $P < 0.0001$) and are thus impaired in olfactory processing. At higher concentrations (10^{-2}), all substances are perceived as repellents, resulting in negative response indices (RI). At this concentration, the response of GH146/TNT flies is unimpaired for three odors tested. For 2-hexanol, however, we find a significant difference at 10^{-2} (Kruskal–Wallis, H = 22.2; $P < 0.0001$). Error bars: ±SEM.

posterior brain (Fig. 1 *B* and *F*), and (*v*) a single descending interneuron with arborizations in the leg neuromeres (not shown). Expression occurs also in glial cells along all major peripheral nerves (Fig. 1 *A* and *I*), as shown by the binding pattern of the glial-specific Repo antibody (28) (data not shown). We note the complete absence of label in olfactory and gustatory receptor neurons.

Responses to Odors. To assess an olfactory function of PNs, we used the T-maze assay, which allowed us to test the behavioral responses of GH146 flies expressing TeTxLC to different odors. Flies were given the choice between different test substances (ethyl acetate, 1-octen-3-ol, 2-hexanol, trans-hexenal) and paraffin oil as a control (Fig. 2). All four chemicals were previously shown to elicit behavioral and/or electrophysiological responses (29–31). For all tested odorants, flies expressing TeTxLC (GH146/TNT) were impaired in odorant detection when tested with lower (10^{-3} or 10^{-4}) concentrations, as their response indices were significantly different from controls. The same effect was observed at higher concentration (10^{-2}) for 2-hexanol but not the other odorants. We conclude that GH146/TNT flies are unable to correctly process odor information at lower concentrations and thus show impaired experience-independent olfactory behavior.

Responses to Sucrose. Studies have postulated the presence of projections from the gustatory centers of the suboesophageal ganglion via the antennocerebral tract to the MB calyx (14, 32). Although line GH146 shows no reporter gene expression in such a pathway (see above), we investigated the ability of GH146/TNT flies to respond to gustatory signals by using the proboscis extension assay. The responses to different sucrose concentrations are shown in Fig. 3. TeTxLC expression clearly had no negative effect on sucrose perception. GH146/TNT flies

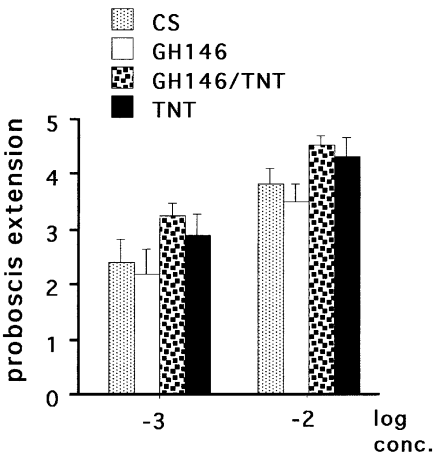

Fig. 3. Proboscis extension responses to gustatory stimulation of the forelegs with sucrose solution of different concentrations (log molarities). Each bar depicts the mean responses of 10 flies to five stimulations. There is a significant effect of genotype (ANOVA, F(3,72) = 4.51, $P = 0.006$). Of all lines tested, GH146/TNT flies respond with the highest frequency for both concentrations and are thus unimpaired in sucrose-induced gustatory processing. Error bars: ±SEM.

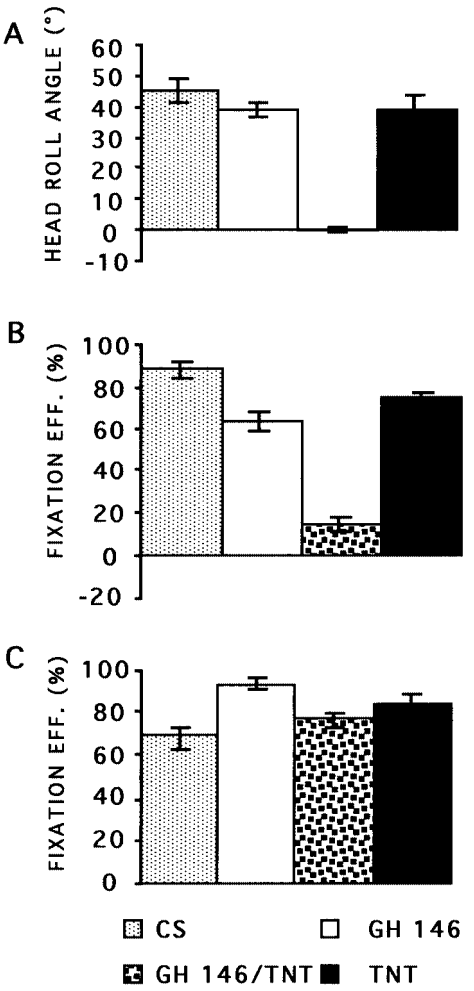

Fig. 4. Visually guided behavior. (*A*) Head roll response to a grating of moving stripes (60° spatial wavelength, 1.2 Hz pattern contrast frequency) is absent in GH146/TNT flies. Each column depicts the mean response of five flies to three stimulations. (*B*, *C*) Fixation efficiencies of freely walking flies toward two opposing 10° stripes (*B*) and 60° stripes (*C*). The mean of 10 flies (each tested 10 times) is given. GH146/TNT flies do not fixate 10° stripes, in contrast to CS, TNT, and GH146 ($P < 0.05$). However, for 60° stripes, GH146/TNT shows fixation behavior, indicating that 60° stripes can be seen by GH146/TNT flies. Error bars: ±SEM.

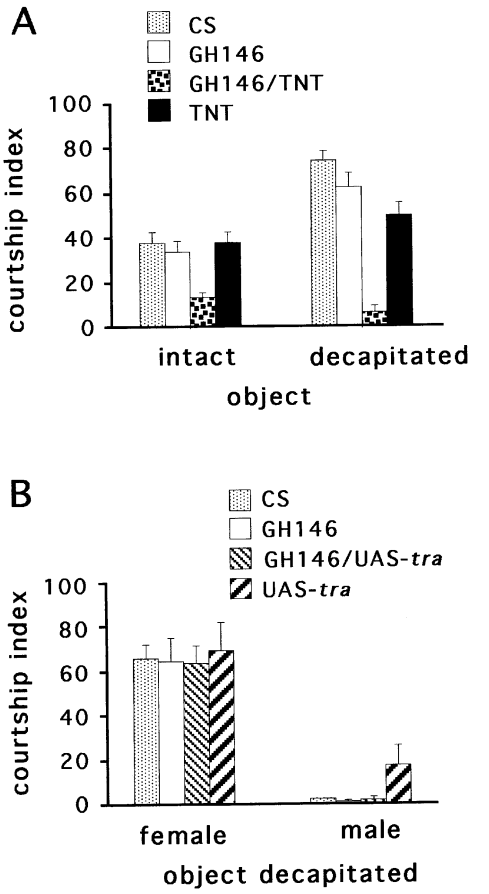

Fig. 5. Male courtship behavior. (*A*) Courtship directed toward intact or decapitated CS female targets represented as a CI. GH146/TNT males display reduced courtship for both intact and decapitated objects (Kruskal–Wallis, intact object: $H = 11.69$, $P = 0.0085$; decapitated object: $H = 44.88$, $P < 0.0001$). All experiments were done by using a red light filter to prevent visual input. (*B*) Female and male targets elicit normal courtship when the same neurons are feminized via ectopic *tra* expression (GH146/UAS-*tra*). Each column represents the mean of 20–29 tests in *A* and 6–11 tests in *B*, respectively. Error bars: ±SEM.

achieved the highest mean response (3.95) when compared with wild-type CS (3.10) and the parental lines GH146 (2.85) and TNT (3.60). We conclude that the neuronal circuit labeled in line GH146 is not necessary for this type of gustatory response.

Visually Guided Behavior. To study the function of TeTxLC-expressing neurons in the visual system (see above), we investigated visually induced head roll and landmark fixation in walking. GH146/TNT flies showed no head roll response to a grating of moving stripes (60° pattern wavelength, 1.2 Hz contrast frequency) (Fig. 4*A*). In addition optomotor responses in walking were absent for two different stimuli (24° pattern wavelength, 3 Hz contrast frequency, and 360° pattern wavelength, 0.2 Hz contrast frequency), and GH146/TNT flies show no visually induced landing response (data not shown). Regarding landmark fixation, GH146/TNT flies were tested for their ability to detect stationary objects of different width. When 10° stripes were presented, GH146/TNT flies walked randomly, whereas CS, GH146, and TNT controls showed normal fixation behavior (Fig. 4*B*). However, when the stripe width was extended to 60°, GH146/TNT flies walked toward the stripes as often as CS, TNT, and GH146 controls (Fig. 4*C*). We conclude that TeTxLC expression in GH146 leads to a complete abolishment of visual behaviors that require motion detection, whereas landmark fixation is only partially impaired.

Courtship Behavior. Male courtship is triggered by various sex-specific sensory cues, including visual and chemical stimuli (24, 33, 34). Some of the neural elements responsible for this complex behavior have been described (18, 35, 36). We addressed the question whether GH146/TNT males exhibit altered courtship performance. Courtship tests under white light showed a strongly reduced CI for GH146/TNT males (data not shown). As this effect could be because of visual and/or olfactory impairments, we tested courtship behavior also under red light, to avoid visual input (Fig. 5*A*) (23). To exclude reciprocal courtship, we additionally used decapitated females (18). Under these conditions, males should largely rely on chemosensory cues. Decapitated and intact females elicited very reduced CIs from GH146/TNT males compared with controls. The low courtship levels were because of a general "disinterest" in the majority of these males, rather than to courtship breakoff after normal onset. However, locomotor activity of GH146/TNT males, measured

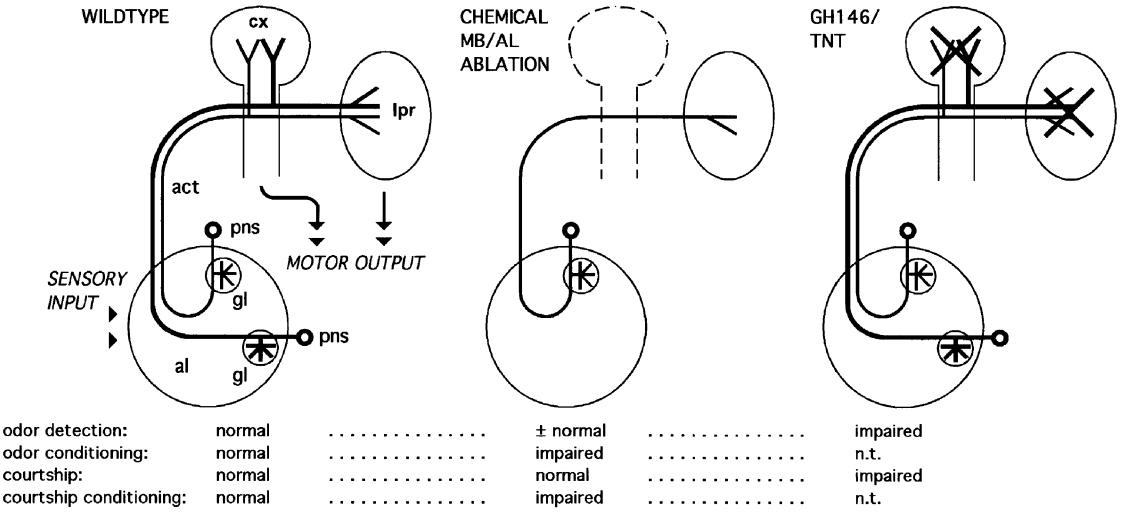

Fig. 6. Schematic representation of different PN target regions and their role in olfactory behavior. In MB/AL ablated flies, a cluster of PNs lateral to the AL (al), and collaterals of the persisting PNs in the MB calyx are missing (16), whereas the direct PN pathway to the LPR remains intact. Such males show normal experience-independent olfactory responses (''odor detection'') (15) and normal experience-independent courtship behavior (''courtship'') (40). However, experience-dependent modulations of olfactory responses (''odor conditioning'') and of courtship behavior (''courtship conditioning'') are impaired. When blocking synaptic transmission in GH146/TNT, information processing via the direct and indirect pathway is largely impaired. As a consequence, these flies are impaired in odor detection and courtship. These results suggest that the direct pathway form the AL to the LPR is sufficient and necessary for experience-independent odor detection and courtship, whereas the indirect pathway is necessary for the experience-dependent modulation of these behaviors. For abbreviations, see Fig. 1.

as the crossing frequency of a center line in the courtship chamber, was not significantly different from wild-type CS (ANOVA, $P = 0.28$, data not shown). We conclude that GH146/TNT males show impaired experience-independent courtship.

To understand whether the circuit labeled in line GH146 includes sexually dimorphic neural elements, we feminized the cells in male flies by using the UAS-*tra* reporter (18). These chromosomally male flies courted decapitated female targets normally and were not attracted by decapitated male flies (Fig. 5*B*). Hence, we find no evidence of sexual dimorphism.

Discussion

We report that TeTxLC expression in line GH146 leads to impaired experience-independent olfactory processing and male courtship. Furthermore, visually guided behavior is altered. TeTxLC expression has been shown to very efficiently block neurotransmitter release at concentrations below the detection limit of the avidin–biotin immunocytochemical method used in our study (37, 38). We therefore presume that we completely interrupted the neuronal pathway labeled in GH146/TNT flies. Because olfactory tests were done under conditions that prevent visual input, we believe that the behavioral effects are largely because of TeTxLC expression in PNs and thus an interruption of olfactory processing. We will first discuss the role of the two PN target regions for these behaviors (Fig. 6).

Previously, it was shown that one can chemically ablate the MBs in the first instar larva (15) together with a subset of PNs (16). The persisting PNs do not form output collaterals in the MB calyx region, whereas their arborizations in the LPR remain intact (16). Experience-independent responses to a number of odors were normal in these flies, but they were impaired in a conditioning paradigm, which requires experience-dependent modulation of olfactory behavior (15). Similarly, such males perform normally in courtship but show poor performance in assays that require experience-dependent modulation (39, 40). Because our study in addition shows that blocking both direct and indirect pathways to the LPR leads to impaired experience-independent olfactory behavior, we propose that the direct pathway is necessary and sufficient for experience-independent olfactory processing (Fig. 6). This assumption predicts that flies with an intact indirect pathway but with compromised function of the direct one should still be defective in experience-independent olfactory processing. However, the tools to perform this experiment need to be developed. As a working hypothesis, we suggest that the LPR integrates both experience-independent and -dependent olfactory processing, the latter via the MB loop. Future studies will show to which extent this generalization is correct.

We detect behavioral differences between GH146/TNT flies and control flies only at low odor concentrations. The normal performance at high odor concentrations could be explained by processing via the remaining noninhibited PNs and projections via other pathways to the LPR (14). It will be important to test whether GH146/TNT flies are able to discriminate among odorants at high concentrations (19).

Expression of TeTxLC in two types of lamina monopolar cells and in unidentified neurons arborizing in the medulla, lobula, and lobula plate led to a complete absence of the behavioral response to movement. However, responses to stationary objects partially persisted. This phenotype is consistent with the functional block of lamina monopolar cells in line GH146. The structural brain mutant *vam* shows a similar anatomical and behavioral phenotype (41). Hence, the visual neurons manipulated in our study are necessary for motion detection but not for the detection of stationary objects.

Males that are defective in movement detection were shown to display subnormal courtship (24, 42). For this reason, we tested courtship behavior under red light, excluding visual cues. The CI reduction thus observed can be attributed to the silencing of the nonvisual elements labeled by GH146. PNs are certainly the most likely candidates to be involved in this behavior. Block of MB function and of the indirect PN pathway should be of no consequence, because MB ablated males perform well in naive courtship (see above) (Fig. 6). A possible role of the few bilateral and descending GAL4-positive interneurons cannot be excluded, although it seems unlikely that the different levels of the courtship pathway should rely on single neurons.

Chemosensory cues are known to play an important role in courtship behavior (34). Sexual pheromones in *Drosophila* are thought to be detected mainly by direct contact, involving gustatory organs on the forelegs and proboscis (43, 44). GH146/TNT performs normally in the sucrose-induced proboscis extension assay, demonstrating that the neuronal circuit affected is not involved in this type of taste response. Although we cannot exclude that PNs may participate in the processing of other gustatory cues—for example, cuticular pheromones—we consider it unlikely because of the lack of obvious gustatory input pathways to PNs. Hence, the reduced CI of GH146/TNT males suggests that PNs may be involved in the processing of volatile pheromones.

Unlike for some other insect species (45), no sexual dimorphism of AL structure or PN identity is described for *Drosophila.* However, feminization of parts of the AL and the MBs, by using the UAS-*tra* construct, resulted in altered sexual orientation (18, 36). We therefore tested courtship performance of GH146/UAS-*tra* males. These feminized males showed normal courtship responses toward females and were not attracted by males. This might seem surprising, because Ferveur *et al.* (18) reported enhanced male courtship toward males in which certain AL glomeruli had been feminized. A clonal analysis (27) has confirmed arborizations of PNs in all of these glomeruli in line GH146. However, these results may not be inconsistent, because different cell types might be labeled in the P[GAL4] lines studied by Ferveur and coworkers. Our results suggest that none of the neurons feminized in line GH146 have to be of male genotype for the normal sexual orientation of the flies. However, the abolishment of courtship in males expressing TeTxLC suggests a crucial role of the direct PN pathway in the processing of volatile pheromonal cues.

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