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Gustatory Stimulation of a Homeotic Mutant Appendage, *Antennapedia*, in *Drosophila melanogaster*

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Summary. The proboscis extension response was used to prove the leg identity of chemosensory neurons in the homeotic appendage of the *Drosophila* mutant *Antennapedia* (*Antp*^{73b}). The data suggest that the homeotic appendage, which is morphologically characterized as a mesothoracic leg, corresponds to a mesothoracic leg as well when considering its gustatory responsiveness (Figs. 1A, B; 3A, B). The neuronal pathway which might mediate the reflex between homeotic chemoreceptors and motor neurons responsible for the proboscis extension is discussed.

1. Introduction

The replacement of arthropod appendages by appendages of a different body region is known from heteromorphic regenerates and homeotic mutations. Heteromorphic regeneration is induced by amputation or loss of specific body parts and may be due to nervous interaction (Borchardt, 1927). In homeotic mutations, on the other hand, the development of an appendage at a site normally occupied by a different appendage is genetically fixed (for review see Gehring and Nöthiger, 1973).

Both heteromorphic regenerates and homeotic mutations raise interesting questions about neuronal specificity. How do the sensory cells of this "ectopic" cuticle connect with the central nervous system? This problem may also be studied by means of surgical experiments, in which sensory epidermis is transplanted to "ectopic" sites. In insects such sensory neurons establish connections in the central nervous system according to the type of epidermis from which they are derived rather than according to their actual position (Palka and Schubiger, 1975). Studies of crayfish heteromorphic regenerates revealed, in contrast, considerable wandering of sensory fibers within the central nervous system, only a small portion of the afferent axons reaching similar central positions as in the transplantation experiment (Sandeman and Luff, 1974).

In a previous paper (Stocker et al., 1976) we have studied the sensory projec-

tion of a homeotic appendage in the mutant *Antennapedia* ($Antp^{73b}$) of *D. melanogaster*, where parts of the antenna are transformed into parts of a leg. Using degeneration technique we were able to demonstrate that the sensory axons of the homeotic leg did not terminate in normal leg projection areas, but within normal antennal centers. Thus in this case sensory neurons seem to behave according to their position than according to their origin. The interpretation of these findings is complicated when considering the behavioral results obtained by Deak (1976) in the homeotic mutant *spineless-aristapedia* (ss^a) of *D. melanogaster*. He observed that the proboscis extension response (PER) usually evoked by stimulation of the tarsi of a hungry fly with a sugar solution (Minnich, 1922) could likewise be elicited by stimulation of the homeotic tarsi.

The present study was undertaken in order to elucidate the relations between our recent neuroanatomical study in $Antp^{73b}$ and Deak's behavioral experiment in ss^a. First, second, and third legs of wild-type and *Antp* flies were tested for PER with sugar solutions of various concentrations and compared to the PER elicited by stimulation of wild-type and *Antp* antennae.

2. Materials and Methods

Antennapedia flies of the genotype $Antp^{73b}/TM_3SbSer$ were used in this study. For explanations of gene symbols see Lindsley and Grell (1968). The phenotype of the homeotic leg was described by Stocker et al. (1976). As control wild-type ("Sevelen") stock with normal antennae was used. *Antp* and wild-type stocks were kindly supplied by G. Schubiger (University of Washington, Seattle, USA).

Only three days old wild-type and *Antp* flies were used for the experiments. The proboscis extension procedure was carried out according to the method of Deak (1976) with some modifications: Flies were starved for 24 h in glass tubes containing moist cotton wool. They were then lightly anaesthetized with CO_2 and attached by the dorsum to a microscope slide by sticking the wings with small pieces of adhesive tape. Animals were then allowed to recover from the anaesthesia for two hours.

Previous to the experiment flies were satiated with water in order to exclude a behavioral response to water stimulation alone (Shiraishi and Tanabe, 1974). This was performed in the usual manner (cf. Getting, 1971; Deak, 1976), i.e., flies were allowed to drink water until no proboscis extension could be elicited by water stimulation of the prothoracic tarsi. Single appendages were then tested for the proboscis extension response (PER) by applying small drops of sugar solutions (sucrose or fructose) during one second to the ventral side of the tarsi. Meso- and metathoracic legs as well as wild-type and *Antp* antennae were tested after sticking prothoracic legs to the slide surface similarly as wings. For removal of the test solutions tarsi were dipped into water and wiped dry with a piece of filter paper. Sufficient time was allowed between successive stimulations in order to exclude habituation and/or facilitation (cf. Deak, 1976). Each test consisted of five consecutive applications of the same solution at approx. 20-s intervals to one of the selected appendages. Various concentrations were applied in a random sequence using right and left appendages indiscriminately. In homeotic leg pairs, however, only one appendage showed usually an optimal and thus usuable phenotype (Stocker et al., 1976). Complete water-satiation of the flies was frequently checked during the experiment. Animals that died during the procedure or within 30 min after the end of the experiment were discarded.

Whole-mounts were prepared of all homeotic legs stimulated with sucrose. Numbers of all bristles and of certain chemosensory hairs (see Results) were counted and compared with corresponding numbers on *Antp* mesothoracic legs, because the $Antp^{73b}$ antenna corresponds morphologically to a mesothoracic leg.

Arithmetic means were calculated from the collected proboscis extension data. Significance was tested using a χ^2-test.

$$\chi^2 = \frac{(r_1 - n_1 \hat{p})^2}{n_1 \hat{p}(1-\hat{p})} + \frac{(r_2 - n_2 \hat{p})^2}{n_2 \hat{p}(1-\hat{p})}.$$

For this purpose the numbers r of flies showing three or more responses in the five trials were determined for calculation of the relative frequency $\hat{p}$ of the experiments with three or more responses in both samples together. n represents the total number of flies used in a test.

$$\hat{p} = \frac{r_1 + r_2}{n_1 + n_2}$$

Results were considered significantly different at $P < 0.005$.

3. Results

The total of proboscis extension experiments carried out is presented in Figures 1 to 3. Since no significant sexual differences were observed, male and female data are pooled. It should be mentioned that the arithmetic means indicated do not necessarily represent average numbers of proboscis extensions; when stimulated five times successively and independently most flies extend their proboscis either five times or do not react at all. Animals responding once, twice, three or four times are less numerous at each concentration. In both wild-type and *Antp* thoracic legs the duration of contact between the sugar solution and the tarsi needed for a proboscis extension response (PER) does usually not exceed half a second. The proboscis is extended fully and rapidly.

Figure 1 A shows the means of PER in wild-type ("Sevelen") flies when stimulated with sucrose solutions of various concentrations. Prothoracic tarsi are by far most sensitive to sucrose compared to meso- and metathoracic tarsi. PER are elicited by a concentration as low as 9.77×10^{-5} M. A maximum mean of 4.64 is obtained at 10^{-1} M. The sensitivity threshold of mesothoracic tarsi is of the magnitude of 100 times higher, at 6.25×10^{-3} M. At the highest concentration tested (4×10^{-1} M) their mean response is 2.87, i.e., about 63% of the prothoracic response. Metathoracic tarsi are almost unresponsive to stimulation, even at very high concentrations. The very few PER observed after stimulation of normal antennae cannot be explained so far.

Figure 1 B shows the same data in *Antp* flies. Pro- and metathoracic data are not significantly different from the corresponding wild-type data ($P > 0.005$). Mesothoracic tarsi are more responsive to sucrose solutions than the corresponding wild-type tarsi. Differences are significant at concentrations 6.25×10^{-3} M and 10^{-1} M.

When homeotic tarsi are stimulated the PER can clearly be evoked (Fig. 1 B). However, flies extend their proboscis less rapidly, less completely, and need a stimulation time of approx. one second. The homeotic tarsi are significantly less responsive at each concentration tested than the morphologically comparable mesothoracic tarsi (cf. Stocker et al., 1976). A maximum of 59.2% of the mesothoracic mean is obtained at 4×10^{-1} M. The lower homeotic data are due to both more unresponsive and low responsive flies than when stimulated on the mesothoracic leg. It was observed several times that the PER could be elicited five times successively by stimulation of the mesothoracic tarsi whereas

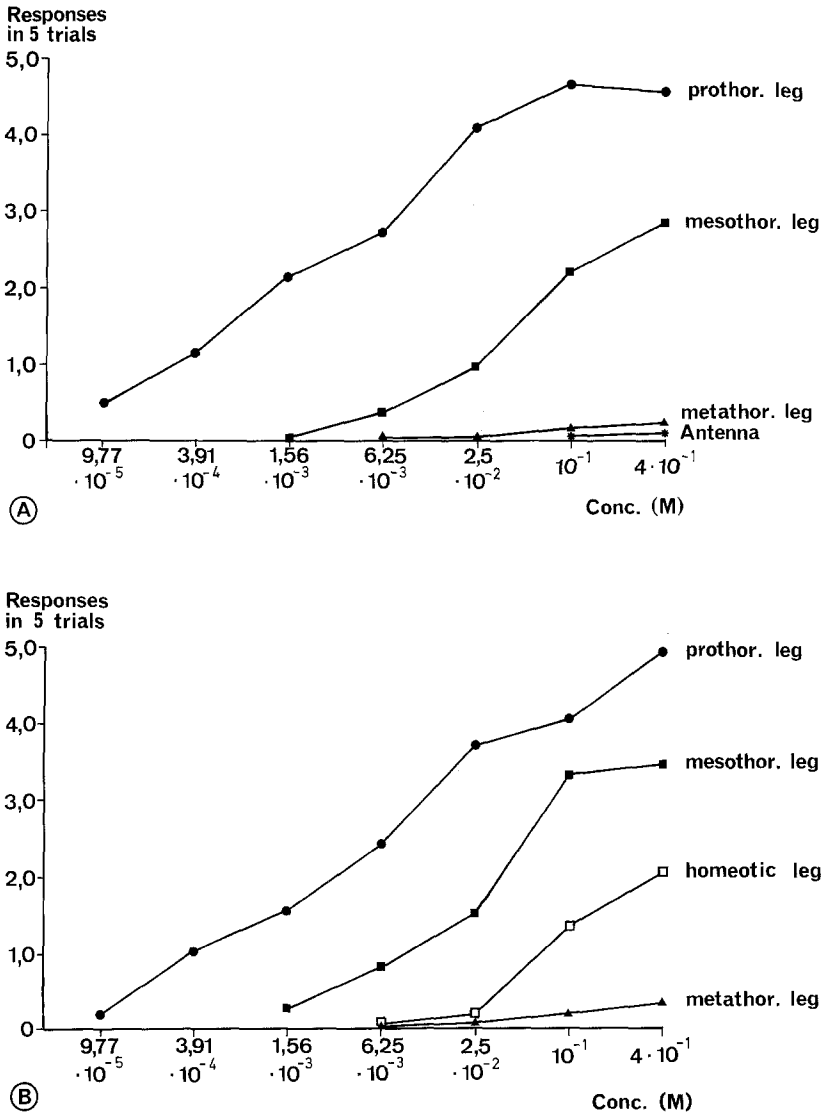

Fig. 1A and B. Arithmetic means of proboscis extensions elicited by 5 successive stimulations with sucrose solutions of various concentrations. ● prothoracic tarsi; ■ mesothoracic tarsi; ▲ metathoracic tarsi; □ homeotic tarsi; * antennae. Each point is based on a minimum of 40 flies. **A** Wild-type ("Sevelen") flies. **B** *Antp*^{73b} flies

homeotic tarsi of the same fly were not responsive at all. The reverse situation was not found.

At this point the question arises as to the factors that might be responsible for the different sensitivities observed in pro-, meso-, and metathoracic legs, as well as in *Antp* mesothoracic and homeotic legs. In order to test whether differing numbers of chemoreceptors might be critical, in a preliminary series of experi-

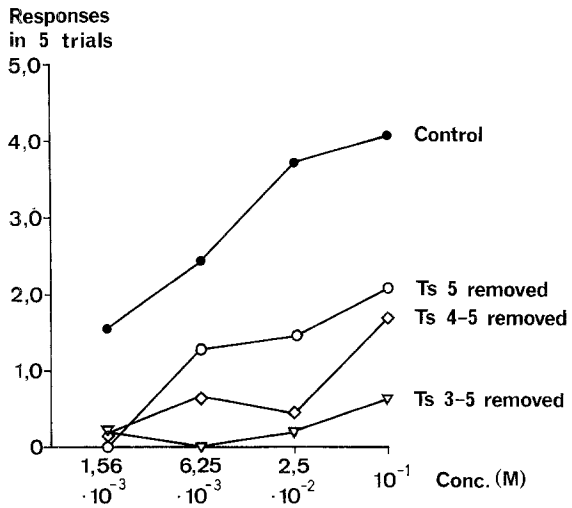

Fig. 2. Arithmetic means of proboscis extensions elicited by 5 successive stimulations of $Antp^{73b}$ prothoracic tarsi with sucrose solutions. One to 3 tarsal segments were previously amputated unilaterally. ● control side; ○ tarsal segment 5 removed; ◇ tarsal segments 4–5 removed; ▽ tarsal segments 3–5 removed

ments one to three tarsal segments were amputated unilaterally in *Antp* prothoracic legs. The stump was then tested with sucrose for PER and compared to responses obtained by stimulation of the control leg (Fig. 2). At concentrations 10^{-1} M and lower even the loss of one segment (Ts 5) results in a substantial decrease of the mean response, caused both by unresponsive and less responsive flies. Thus at concentrations tested the PER depends heavily on the number of chemoreceptors stimulated.

In order to elucidate the differences in responsiveness between *Antp* mesothoracic and homeotic legs, the numbers of all bristles (hairs and spines) and of certain readily identifiable gustatory hairs (see below) on the tarsal segments of both appendages were counted and compared (Tables 1 and 2). Since no significant sexual variations were observed, male and female data are pooled. With the exception of the first segment the bristle number of homeotic tarsal segments is smaller than that of the corresponding mesothoracic segments (Table 1). Especially the distal segments 3, 4, and 5 are very low in numbers and

Table 1. Total bristle number in $Antp^{73b}$ mesothoracic and homeotic tarsal segments

		Tarsal segments					Total	*n*
		1	2	3	4	5		
Mesothoracic leg	Means	66.3	35.3	20.2	18.4	19.7	159.9	10
	%	100.0	100.0	100.0	100.0	100.0	100.0	
Homeotic leg	Means	69.2	27.5	13.7	13.0	13.4	136.8	51
	%	104.3	78.0	68.0	70.7	67.8	85.6	

Table 2. Numbers of certain gustatory hairs (for description see text) in $Antp^{73b}$ mesothoracic and homeotic tarsal segments

		Tarsal segments					Total	*n*
		1	2	3	4	5		
Mesothoracic leg	Means	6.6	3.5	0.6	2.6	4.0	17.3	10
	%	100.0	100.0	100.0	100.0	100.0	100.0	
Homeotic leg	Means	7.0	2.7	0.2	1.9	2.0	13.9	51
	%	106.7	78.4	29.4	72.4	51.0	80.4	

obtain only 68.0%, 70.7%, and 67.8% respectively of the mesothoracic means. On an average homeotic tarsi bear 14.4% less bristles than mesothoracic tarsi.

The relation between homeotic and mesothoracic tarsal segments in *Antp* is even more pronounced when considering the numbers of certain gustatory hairs (Table 2). The hair type counted is slender and curved and tends to project from the surface of the tarsus farther than the thick, straight and sharp-tipped spines. The latter are presumably not innervated (Grabowski and Dethier, 1954). In contrast to the spines the tip of these hairs is blunt characterizing them as gustatory sensilla (Grabowski and Dethier, 1954). It is not clear to which of the four morphologically distinct tarsal chemoreceptors of the blowfly this hair type corresponds (Grabowski and Dethier, 1954; Hansen and Heumann, 1971).

Whilst the first homeotic tarsal segment has slightly more of these hairs than the corresponding mesothoracic segment, hair numbers counted on all other tarsal segments are again much lower in the homeotic leg (Table 2). The third segment bears 70.6% less, the fifth segment 49.0% less hairs than the corresponding mesothoracic segments. On an average homeotic tarsi bear 80.4% of the hair type counted compared to mesothoracic tarsi. These data imply that the homeotic leg nerve contains less axons originating from tarsal chemoreceptors than the mesothoracic leg nerve, the sensory input to the CNS being obviously smaller, too.

In order to test whether the homeotic chemoreceptors respond with the same specificity as the mesothoracic ones, a second series of experiments was performed using fructose solutions. The data obtained from stimulation of wild-type tarsi and antennae (Fig. 3A) are very similar to those obtained from sucrose experiments (Fig. 1A), although pro- and mesothoracic tarsi seem to be slightly less responsive to fructose. Differences significant from the corresponding sucrose data are at concentrations 9.77×10^{-5} M, 3.91×10^{-4} M, and 1.56×10^{-3} M (for prothoracic legs) and 10^{-1} M (for mesothoracic legs).

The contrast of sucrose and fructose tests is even more pronounced in *Antp* thoracic tarsi (Figs. 1B and 3B respectively) than in wild-type comparisons. Especially at low concentrations up to 2.5×10^{-2} M prothoracic tarsi are significantly less responsive to fructose than to sucrose. Mesothoracic fructose data are equally lower at low concentrations, but are not significantly different.

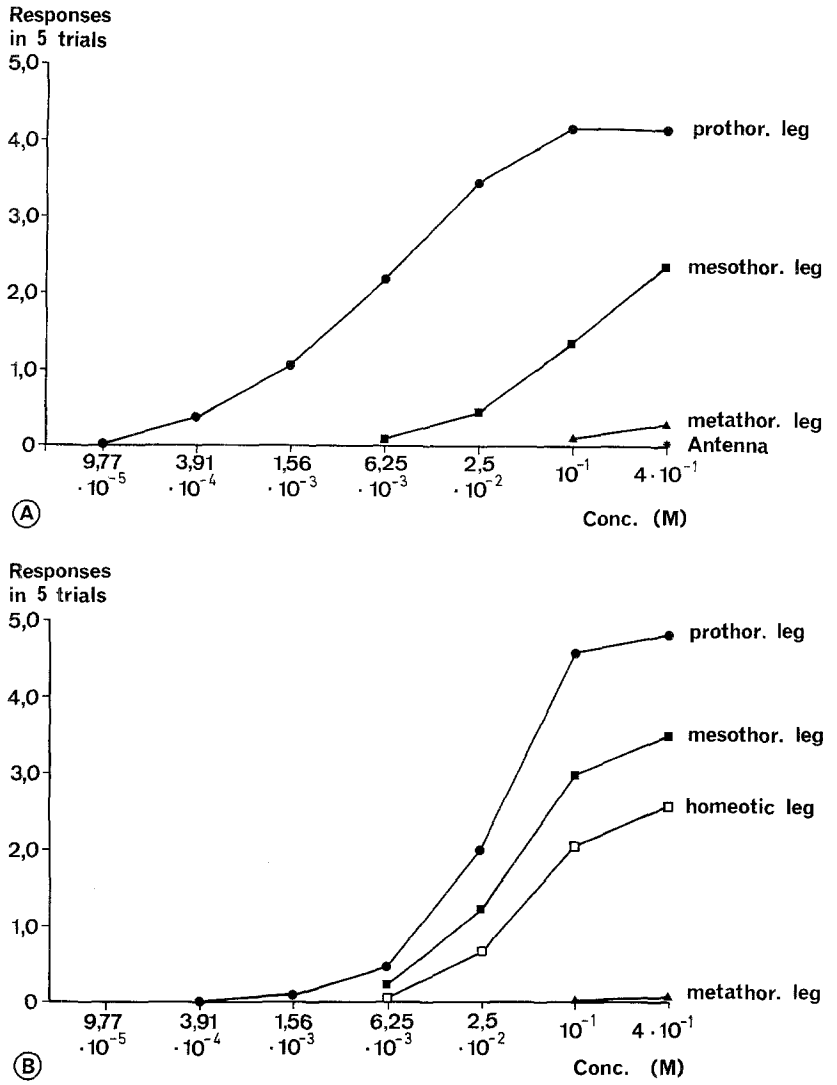

Fig. 3A and B. Arithmetic means of proboscis extensions elicited by 5 successive stimulations with fructose solutions of various concentrations. ● prothoracic tarsi; ■ mesothoracic tarsi; ▲ metathoracic tarsi; □ homeotic tarsi; * antennae. Each point is based on a minimum of 40 flies. **A** Wild type ("Sevelen") flies. **B** $Antp^{73b}$ flies

Comparison of wild-type and *Antp* fructose experiments reveals significantly lower sensitivity of *Antp* prothoracic tarsi at concentrations up to 2.5×10^{-2} M, whereas mesothoracic data of *Antp* are significantly higher at 10^{-1} M.

PER are more readily evoked when stimulating homeotic tarsi with fructose than with sucrose solutions. This difference is significant at 10^{-1} M. The means calculated from fructose stimulation of homeotic tarsi are lower than those obtained by fructose stimulation of the morphologically related mesothoracic

tarsi, but differences are below significance at concentrations 2.5×10^{-2} M, 10^{-1} M, and 4×10^{-1} M. The responsiveness of *Antp* flies stimulated on either homeotic or mesothoracic tarsi is thus much more similar in the fructose than in the sucrose experiment.

4. Discussion

The data presented clearly demonstrate that in $Antp^{73b}$ flies a proboscis extension response (PER) can be elicited by gustatory stimulation of thoracic as well as homeotic tarsi. Since this behavior cannot be evoked by gustatory stimulation of normal (wild-type) antennae, our observations are proof of the legness of the chemosensory neurons in the transformed appendage. An identical experiment has recently been performed in $Antp^{B}$ flies (Deak, 1976), but with negative result. This failure was explained by missing tarsal segments of the homeotic appendage used. However, in the same paper this author demonstrated the presence of leg-like epidermis in homeotic appendages of the mutant *spineless-aristapedia* (ss^{a}) in *D. melanogaster*.

Considering the quantitative aspects of our data it is obvious that pro-, meso-, and metathoracic tarsi differ in their responsiveness to sugar solutions, in that prothoracic tarsi are most responsive and metathoracic tarsi almost unresponsive, mesothoracic tarsi taking an intermediate position. This is true for both wild-type ("Sevelen") and *Antp* thoracic legs tested either with sucrose or fructose. These variations provide a favorable tool for specification of leg epidermis.

Differences in responsiveness could be attributed to different sensitivities of the sensory neurons involved, or to variations in the central circuitry. Neither of these interpretations has been followed in this paper. It has been observed, however, that the size of the PER is strongly dependent on the number of gustatory hairs stimulated. This is demonstrated by the significantly lower responsiveness of *Antp* flies stimulated on prothoracic legs after amputation of various tarsal segments. Counts of tarsal chemosensory hairs in blowflies have revealed large differences between thoracic legs, the relations of pro-, meso-, and metathoracic tarsi being approx. 3:2:1.5, respectively (Grabowski and Dethier, 1954). In the present study no detailed counts of tarsal chemosensory hairs were performed in the three thoracic legs of *Drosophila*, but a rough comparison supports the observations made in blowflies.

Arrangement and numbers of bristles characterize the transformed antenna of the $Antp^{73b}$ mutant as a mesothoracic leg (Stocker et al., 1976). The proboscis extension data presented here suggest that this relation might be valid at the physiological level as well. The lower responsiveness of homeotic tarsi compared to mesothoracic tarsi may be mainly explained by lower numbers of chemoreceptors counted in the homeotic tarsi, and thus a smaller sensory input to the CNS. However, as mentioned above, it cannot be excluded a priori that the sensitivity of the chemoreceptors itself is lower than in mesothoracic tarsi, or that the differences are due to the changes in the central circuitry.

A previous neuroanatomical study in $Antp^{73b}$ has shown that the sensory

axons of the homeotic leg project into normal antennal centers, i.e., both ipsi- and contralateral antennal glomeruli (Stocker et al., 1976). Several interpretations of these results have been proposed. Based on the presence of homeotic axon terminals in antennal centers and their absence in leg centers it has been suggested that sensory neurons in the homeotic appendage might be antennal in identity. Considering our behavioral results this possibility seems very unlikely, since antennal chemoreceptors are not responsive to sugar solutions. It would be surprising if homeotic receptors other than gustatory ones would have retained their antennal expression, since the transformation of the antennal disc into a leg disc occurs in the third instar (Postlethwait and Schneiderman, 1971), before the developmental separation of the sensory neurons into different modalities.

The behavioral data presented above tend to characterize the homeotic projection as "wrong", since homeotic axons do not terminate in areas where normal leg axons do. How this "wrong" projection pattern might occur is discussed in detail elsewhere (Stocker et al., 1976).

The key attention regarding neuroanatomical and behavioral results is focused on the question how the PER can be elicited in spite of the "wrong" projection of homeotic fibers. In order to understand how the central circuitry might have been adapted to this new projection pattern one has to consider the normal route which action potentials generated in leg chemoreceptors follow through the CNS to the motor neurons which cause the proboscis to extend. Unfortunately our knowledge about this question is still rather limited.

Recent neuroanatomical investigations suggest that higher brain centers might be involved in mediating this reflex behavior. In muscids labellar chemosensory axons synapse with first order interneurons in the suboesophageal neuropil which extend into the tractus olfactorio-globularis and thence into the mushroom bodies (Strausfeld, 1976). Tarsal chemoreceptors, on the other hand, are known to synapse with first order interneurons in thoracic ganglia (Geisert and Altner, 1974; Stocker et al., 1976). Again some of these interneurons send axons via the suboesophageal ganglion and the tractus olfactorio-globularis to the mushroom bodies (Strausfeld, 1976). According to this author there is good evidence for supporting the idea that information processing of gustatory input is mainly mediated by the mushroom bodies. Descending tracts connecting this association center with motor neurons in the suboesophageal ganglion might then close the reflex arc.

However, it cannot be excluded that other local centers for taste are present in the CNS, without the mushroom bodies as an intermediary station (Strausfeld, 1976). Based on the peripheral nerves involved in the PER when labellar chemosensory hairs are stimulated, Dethier (1959) suggested that central nervous elements utilized in this reflex are restricted to the suboesophageal ganglion. In this context it would be interesting to known whether the first order thoracic interneurons mentioned above alternatively branch and synapse with proboscis extension motor neurons in the suboesophageal neuropil.

A neuronal model of the PER elicited by stimulation of labellar chemosensory hairs was proposed by Dethier et al. (1965) and confirmed by Getting (1971). It involves at least one interneuron, but the presence of a second one

is assumed on the grounds of the interplay of the gustatory system with other sensory modalities.

When discussing the reflex pathway originating in homeotic tarsi one has to consider that mushroom bodies are likely to represent olfactory as well as gustatory association centers (Strausfeld, 1976). They receive olfactory input from the antennal glomeruli via first order interneurons which pass through the same tractus olfactorio-globularis as the gustatory interneurons mentioned. In addition, some so-called "long olfactory fibers" have been followed from muscid antennae directly into the mushroom bodies (Strausfeld, 1976). Remarkably enough these by-passing sensory fibers have been traced in *Drosophila* from both wild-type and *Antp* antennae using cobalt chloride diffusion (Stocker et al., 1976). These fibers, as well as the first order olfactory interneurons might thus provide a pathway between the homeotic chemoreceptors and the gustatory association centers in the brain. Alternatively, since after bilateral amputation of homeotic legs degeneration spots occur in regions near the suboesophageal neuropil (Stocker et al., 1976), it is also possible that the reflex is mediated directly with the suboesophageal ganglion without the participation of higher brain centers.

Assuming either of these models it must be admitted, however, that basic questions remain open. Do for example first order olfactory interneurons change in function to gustatory interneurons? If so, do they form exceptional synapses upon second order gustatory interneurons in the mushroom bodies, or do such synapses also exist in wild-type brains acting as a source of sensory input of different modality? (cf. Dethier et al., 1965). How do the by-passing homeotic fibers find their way to the gustatory interneurons in the mushroom bodies?

There are a few observations which might give some information about the central connectivity in the homeotic PER pathway. No correlation was, e.g., found in $Antp^{73b}$ flies between the responsiveness and the number of gustatory hairs (within certain limits) on the homeotic tarsi stimulated. Thus larger numbers of gustatory hairs on homeotic tarsi do not necessarily yield a better response, and vice versa. Furthermore, when stimulated on homeotic legs *Antp* flies need a longer stimulation time, and the proboscis is less rapidly and completely extended than when stimulated on pro- or mesothoracic legs. After labellar stimulation in *Phormia* comparable responses are obtained only with a very weak stimulus (Dethier, 1959). Similar weak PER as in *Antp* have been reported from ss^a homeotic legs (Deak, 1976). These observations suggest that sensory fibers from homeotic tarsi are less precisely matched with gustatory interneurons than those from thoracic tarsi. A less than optimal pathway in the homeotic PER is to be expected and reflects that the ability of the insect nervous system to adapt to a changed input is not absolute. The presence of the PER generated in homeotic appendages, however, is surprising and demonstrates extensive neuronal plasticity.

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