

Transsexual Limb Transplants in Fiddler Crabs and Expression of Novel Sensory Capabilities

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ABSTRACT

We used transsexual limb transplants in fiddler crabs to examine how peripheral sensory structures interact with the central nervous system (CNS) to produce a sexually dimorphic behavior. Female and male chemosensory feeding claws were transplanted onto male hosts in place of nonfeeding, nonchemosensory claws. Successfully transplanted claws retain donor morphologies and contain chemosensory neurons. Neurons in successfully transplanted female feeding claws express the enhanced sensitivity to chemical cues seen in female, but not male, neurons in claws of normal animals. When chemically stimulated, the transplanted claws evoke feeding behavior not observed in normal males, even though the sensory neurons in the transplanted limb project to the host's sexually dimorphic neuropil not known to receive chemosensory input. Behavioral sensitivity is directly related to the sensitivity of peripheral neurons in the transplanted feeding claw. Thus, the interactions between peripheral neurons and their targets may restructure the CNS so that novel sensory capabilities are expressed, and this can produce sexually dimorphic behaviors. *J. Comp. Neurol.* 440:311–320, 2001. © 2001 Wiley-Liss, Inc.

Indexing terms: sexual dimorphism; limb transplants; fiddler crabs; sensory capabilities; neuronal plasticity

The properties and function of sensory systems depend on characteristics of both the peripheral sensory elements and the central nervous system (CNS). Sensitivity and dynamic range, among other properties of receptor neurons, set limits on responses to physical stimuli such as light, sound, and chemical cues (see, e.g., Atema et al., 1988; Weissburg and Derby, 1995; Lehrer, 1997). Projection patterns of sensory afferents map various stimulus features onto a spatial dimension in many sensory systems (see, e.g., Konishi, 1986; Hayama and Caprio, 1989; Newland et al., 2000). In the CNS, modality-specific organization of the neuropil (see, e.g., Murphey et al., 1989; Killian et al., 1993; Newland et al., 2000) and spatial organization of distinct cell types (Goldsmith, 1991; Meisami, 1991) occur that presumably reflect the need for particular patterns of connectivity necessary for proper sensory functioning. Although it is possible to describe such features as a property of either the CNS or the periphery, in many cases it is the interaction between peripheral sensory elements and their targets that affects the structure and operation of the CNS. This observation

is supported by a variety of transplantation experiments that induce axons to grow into foreign neuropil, which demonstrate that sensory neurons have the capacity to influence patterns of organization in the CNS (Schneiderman et al., 1986; Sur et al., 1990; Scalia et al., 1995; Rössler et al., 1999; Yaka et al., 1999).

It is difficult to assess fully the significance of peripheral effects, because the ability to experimentally link sensory elements with given properties to specific CNS elements is rare, particularly in systems in which corresponding behavioral and physiological studies are possible. Thus, it is fairly difficult to establish the extent to which a peripheral

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Received 3 May 2001; Revised 10 August 2001; Accepted 27 August 2001

structure, either through its specific properties or via its influence on the organization of the CNS, dictates a given level of sensory performance. To examine the extent to which the periphery can legislate behavior, we asked a simple question: Can the transplantation of sexually dimorphic appendages make connections with novel targets in the host CNS to produce behavior normally seen only in the donor organism?

Fiddler crabs (genus *Uca*) are sexually dimorphic crustaceans especially suitable for transplantation studies. Males possess a single hypertrophied chela (the major claw), which is used for communication and defense, and a single feeding (or minor) claw. Females have two feeding claws (Crane, 1975). Chemosensory neurons in female claws express greater sensitivity to chemical stimuli than the neurons in male feeding claws (Weissburg and Derby, 1995; Weissburg, 1999), and this disparity appears, in part, to regulate sex-specific behavioral thresholds observed during feeding (Weissburg, 1993). Chemosensory neurons are known to be present in the feeding claws of males and females; male major claws have never been shown to detect chemical cues in either physiological (Weissburg and Derby, 1995) or extensive behavioral (Valiela et al., 1974; Robertson et al., 1981; Weissburg and Zimmer-Faust, 1991; Weissburg, 1993) investigations.

In this study, we transplanted female feeding claws onto the site of the major claw of host males to determine whether behavioral sensitivity characteristic of females was contingent on the identity of the transplanted limb or the host CNS. Thus, we ask questions concerning both the degree of autonomy expressed by developing limbs and the extent to which neurons in these limbs subsequently influence the ability of the CNS to process novel sensory inputs. We find that, indeed, sensory neurons from transplanted feeding claws retain their characteristic sensitivity, successfully invade foreign neuropil to establish functional connections, and produce behavior that is predictable from the properties of the donor limb.

MATERIALS AND METHODS

Transplantation

The sand fiddler crab, *Uca pugilator*, was used in these studies. Crabs were obtained from a commercial supplier and shipped to our laboratory. There they were held in 45 liter aquaria containing 25 ppt seawater and natural sand substrates. A microprocessor-controlled timer was used to drain and fill the tanks to simulate tidal conditions normally experienced by animals in the field. Donor animals were induced to autotomize feeding claws by squeezing on the merus of the appendage with a forceps. Six walking legs were removed by the same method in order to hasten molting (Hopkins et al., 1999). Thereafter, crabs were kept individually in small bowls with micrometer-filtered, sterile artificial seawater (ASW) media in an incubator at 26°C and a 12:12 hour L:D cycle.

Regenerating crustacean limbs develop through a series of stages into a bud-like structure, the limb bud, during the intermolt period following limb loss (Hopkins et al., 1999). The limb bud matures into a fully formed limb when the animal molts. Following autotomization, we monitored the development of this bud in the donors, and, when it was approximately 2 mm long, animals were transferred to ASW supplemented with 30 mg/liter genta-

micin and 4 ml/liter Fungizone for 3 days prior to surgery. At this same time, host males were size matched with donors and also placed in antibiotic ASW. Immediately prior to surgery, donor and hosts were chilled to quiescence, placed ventral side up, and immobilized with wax. The limb bud of the donor crab was removed at the coxal joint, and the hard cuticle of the ischium was stripped away. Simultaneously, a second person prepared the host crab by inducing autotomy of the major claw and six walking legs, and breaking through the autotomy membrane that seals the wound after limb loss. The bud was carefully transferred to the host and oriented in the socket, using a small amount of cyanoacrylate adhesive to seal the bud onto the host. The host was returned to antibiotic ASW and held in the incubator for another 1 week. Thereafter, the animals were kept in sterile ASW until they molted. An appreciable proportion (see Results) of animals showed apparent regression or lack of development of the transplant, so on the first postsurgical molt only a small, undifferentiated bud (the blastema) was present. These animals were allowed to feed ad libitum for 1 week and then subjected to another round of autotomy of their walking legs to induce a second postsurgical molt.

Using these techniques, we transplanted 448 feeding claws from female donors and 235 feeding claws donated from males. These experiments were performed in the summers of 1998, 1999, and 2000, because animals are available only seasonally.

To facilitate morphological and functional characterization of transplanted feeding claws, we performed a variety of other manipulations to produce regenerating major claws. Transplant controls (sham-operated males) were created by autotomizing the major claw followed by removing the developing limb bud when it reached approximately 2 mm in size. The subsequent molt produced an animal with a developing limb bud on the manipulated side, which is the same condition experienced by animals that failed to develop a claw from a transplanted bud on the first postsurgical molt. In addition, we autotomized major claws and allowed them to regenerate naturally. Table 1 lists the various experimental treatments, the descriptive terms used throughout the text, and a brief summary of their properties.

Neuronatomical studies

In fiddler crabs, all of the nerves emanating from the claw on a given body side project to the ipsilateral first thoracic hemiganglion. The left and right hemiganglia are a bilateral pair of structures that are part of the fused-ring thoracic ganglion, which contains local processing regions corresponding to each of the five limb-bearing thoracic segments.

We performed two different types of anatomical studies on animals receiving transplants. One group of animals was prepared for transmission electron microscopy (TEM) using protocols described fully by Weissburg et al. (1996). The CNS was fixed in situ using a standard crustacean formaldehyde-glutaraldehyde fixative, postfixed in 2% osmium tetroxide, and dehydrated in a graded ethanol series. The hemiganglion receiving the axons from successfully transplanted claws and the contralateral hemiganglion, which receives axons from the native claw, were cleared in propylene oxide and embedded in Araldite-Epon resin. The entire nerve bundle was thin sectioned (thickness 0.075 μm) as it entered the ganglion in order to

TABLE 1. Summary Properties of the Various Types of Transplanted Claws Generated in This Study¹

| Treatment group | N | Description | Physiological and behavioral properties |
|--------------------------|-----|--|--|
| Successful transplants | 101 | Feeding claws from male or female donors that develop into feeding claws on the first or second postsurgical molt when transplanted to the host major claw site | Contain chemosensory neurons and elicit feeding; sensitivity controlled by donor sex |
| Unsuccessful transplants | 31 | Feeding claws from male or female donors that fail to develop into a feeding claw when transplanted to the host major claw site, such that the male regenerates a major claw | No physiological sensitivity detected; do not elicit feeding behavior |
| Transplant controls | 26 | Autotomized major claws removed again during the limb bud stage and then allowed to regenerate | No physiological sensitivity detected; do not elicit feeding behavior |
| Regenerating male claws | 16 | Autotomized major claws allowed to regenerate naturally | No physiological sensitivity detected; do not elicit feeding behavior |
| Native claws | — | The feeding claw (one in males, two in females) normally present in adult crabs | Contain chemosensory neurons and elicit feeding; properties sex-specific |

¹N, number of claws produced. The nomenclature used for treatment group represents the terms used throughout the text to refer to claws of a particular type.

visualize the axons that projected to this neuropil on both the transplanted and the native claw sides. Single photographs of the entire root were taken at low magnification ($\times 200$ – 300) with a Siemens 102 transmission electron microscope.

Dye-tracing studies were performed on three host crabs bearing transplanted feeding claws using the carbocyanine dye DiI protocol developed for crustaceans (Sandeman and Sandeman, 1994). The entire fused-ring thoracic ganglion was removed and fixed using standard protocols (Weissburg et al., 1996). A small crystal of DiI was placed on the cut end of the nerve that contains all the axons from the transplanted claw, and this preparation was allowed to remain in the dark for at least 6 months at room temperature, at which time visual inspections indicated that the DiI had stopped migrating. The specimen was cleared in meglumine iohalamate for easier visualization under light microscopy (Zill et al., 1993). The whole mount was then viewed and photographed on a Zeiss confocal microscope using LSM 510 software. A series of stacked images was taken using a $5\times$ objective, and then each series was collapsed to produce a single two-dimensional image.

Physiology of chemosensory neurons

Methods were used as described by Weissburg and Derby (1995). Briefly, claws were autotomized, stripped of all cuticle proximal to the merus to expose the nerve and artery, and placed into a glass and Teflon olfactometer. The claw was perfused by delivering oxygenated artificial saline into the artery via a glass cannula, and stimuli were supplied using a computer-controlled solenoid valve that injected 0.5 ml of stimulus into ASW flowing over the claw. A fine-tipped, saline-filled microelectrode was attached by suction to axons in an en passant configuration. Chemosensitive neurons were identified using “plant mix” (PM) as a search stimulus. PM is an equimolar solution of five compounds (D-galactose, D-glucose, D-maltose, D-sucrose, and L-serine) that occur in the natural food of fiddler crabs (Darley, 1977). Neurons were presented with PM at concentrations from 10^{-5} to 10^{-1} M, in ascending order, with a 1 min interstimulus interval. Signals were recorded and amplified using conventional AC differential techniques and digitally stored with commercial software (DataWave; Longmount Co.). Recordings typically contained spike waveforms from one to three cells, which were sorted off line into individual units on the basis of various waveform features (rising and falling slope, amplitude, width) using the DataWave software

package. The numbers of spikes were quantified over 4.5 seconds following stimulus introduction and corrected for responses to the ASW control. These techniques were the same as those employed in earlier studies with this animal (Weissburg and Derby, 1995; Weissburg, 1999), which facilitated direct comparisons with the physiological properties of normal male and female chemosensory neurons.

Behavior

A syringe equipped with an 18-gauge needle was used to deliver a single drop of chemical stimulus onto the distal tip of the feeding claw in order to elicit feeding. Five to seven drops (approximately $10\ \mu\text{l}$ each) of a given stimulus were delivered alternately to each claw while the behavior of animals was videotaped under dim red light. Stimuli were ASW and PM at doses from 10^{-3} to 10^{-1} M, presented in random order. The interstimulus interval was 1 minute, contingent on the requirement that animals exhibited no feeding activity for 30 seconds prior to stimulation. Sensitivity of each claw was assessed by reviewing the video records and scoring the frequency with which a given stimulus elicited feeding, defined as occurring when the animal lifted either claw from the sediment surface to its mouth. A given stimulus presentation was excluded from analysis if the video records revealed that the stimulus did not arrive squarely on the distal tip of the claw, or if feeding occurred more than 10 seconds after stimulation. These occurrences were rare, and we selected the first five stimulations that met our criteria for statistical analysis. An individual who was not aware of the presentation order of the stimulus intensities analyzed the video records. The response frequency across individuals cannot be normally distributed because the resulting frequencies fall into a small number of discrete states (e.g., 0, 0.2, 0.4, etc.). Consequently, these data cannot be analyzed by analysis of variance. However, we can account for the pairwise nature of the design by using log-likelihood tests to examine the frequency of responses for an animal's native vs. transplanted side for each experimental group (e.g., animals receiving feeding claws from male or female donors).

RESULTS

Morphology of transplanted claws

Developing limb buds transplanted to the site of a male major claw generated limbs morphologically indistinguishable from normal feeding claws on the first or second

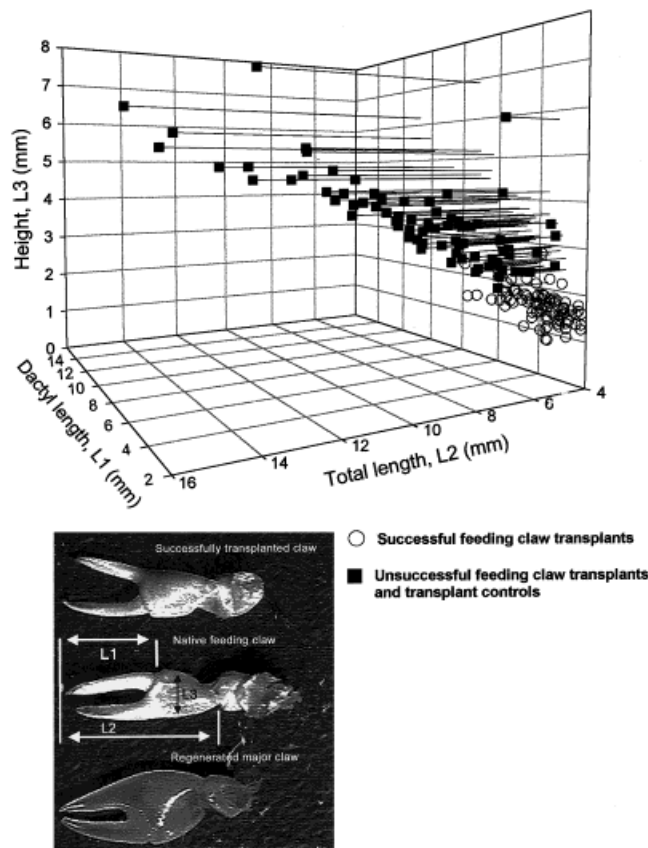


Fig. 1. Morphology of different claw types. **A:** Three-dimensional plot of morphological measurements of different claw types in animals receiving limb transplants. Measurements (shown at bottom) were dactyl (L1) and total claw (L2) lengths, claw height (L3), and claw width (not shown), each made to the nearest 0.01 mm. Note how the claws of successful transplants (circles) cluster in the lower right corner and are morphologically distinct from the claws designated as unsuccessful transplants (squares). For this figure, claws of successful transplants on either their first or subsequent claw-bearing molts were pooled, because initial analysis revealed no discernible difference in claw morphology. Similarly, there was no difference in male major claws from unsuccessful transplants, transplant controls, and newly regenerated male major claws, so these groups also were pooled. Transplanted claws initially were assigned to successful vs. unsuccessful groups if total length (L2) was less than 6.5 mm and height (L3) less than 2.3 mm. This decision was based on a priori inspection of patterns of claw morphology that revealed an apparent gap in the data between groups. A subsequent discriminant function analysis using these measurements (plus claw width) indicates a significant effect of treatment on claw morphology (multivariate $F = 90.00$, $P < 0.001$, $df = 4, 201$), and the resulting function based on these four variables is able to separate transplants from the other pooled treatments with a success rate of greater than 90%. Sample sizes for these groups are given in Table 1. The number of measurements of successful transplants exceeds the number of successful surgeries reported in the text, because animals receiving successful limb grafts frequently survived through several molts. Bottom panel shows morphology of the three types of claws and the measurements used to characterize them. The transplanted feeding and native feeding claws are from a single crab, and the male major claw developed from a male in the transplant control group. The successfully transplanted feeding claw is from a female donor. The native claw is shown upside down, so that the limb is always oriented with its medial face pointing toward the viewer.

postsurgical molt (Fig. 1). These successfully transplanted feeding claws preserved their characteristic form through at least three molts after surgery without showing any indication of transforming into a major claw. From 683 initial surgeries, 281 animals survived through the first molt. Based on our morphological analysis (Fig. 1), 45 of these animals developed a feeding claw and 18 expressed a male major claw. The remaining 217 animals were autotomized again, and those expressing feeding vs. major claws at the next (second) postsurgical molt were 56 vs. 13, respectively. Once established, claw asymmetry is fixed in *Uca*, and the removal of the major claw does not drive the transformation of the contralateral appendage into a major claw, as in snapping shrimp (Govind and Pearce, 1988). As a result, males receiving transplants have two feeding claws, the animal's native feeding claw as well as a second feeding claw from the female or male donor.

The intermolt period in all groups appeared typical for animals receiving multiple limb autotomy when kept under conditions similar those used here (see, e.g., Hopkins et al., 1999). There was no systematic difference in intermolt interval between animals gaining a claw of any type on either the first or the second molt and those that failed to develop a claw on the first molt. Pooled across all animals that survived the molting process (and including animals that molted more than once), the average intermolt period was 22.6 ± 5.8 days (mean \pm standard deviation, $N = 350$).

Neuroanatomical studies

Transmission electron micrographs from the hemiganglion receiving axons from transplanted claws revealed an apparently normal structure of the nerve bundle emanating from the transplant. Similar to our observations of normal animals (Weissburg et al., 1996), the nerve bundle consisted of two roots. The smaller root is characterized by a distinct collection of axons that, based on their number, large diameter, and heavy myelination, appear to emanate from motor neurons (Young and Govind, 1983; Weissburg et al., 1995). The second, larger root (Fig. 2) consisted of mainly small ($<1.0 \mu\text{m}$) fibers characteristic of sensory axons and contained the majority of the sensory afferents. Ganglia from both normal and transplanted animals show a ventral-medial cluster of large, heavily myelinated axons that also may be motor neurons. In most cases, we could discern little difference in the cross-sectional area of the nerve roots entering the ganglia from the animal's transplanted vs. native claws. This stands in contrast to the observations of normal males, in which the nerve root emanating from the major claw has over twice the cross-sectional area of the feeding claw (Young and Govind, 1983). Fasciculation in nerves from the grafted limb sometimes appeared less well developed in comparison to the nerve root from the animal's native side (Fig. 2), although we have observed similar variation in the nerve roots of normal males and females (see, e.g., Weissburg et al., 1996). At least some of the apparent lack of fascicles may reflect the location of the section plane relative to the entrance into the ganglion. These qualitative observations suggest that transplanted structures continue to develop normally, and their neurons innervate the CNS in a manner at least superficially similar to that of the native limb.

DiI fills (Fig. 3) indicated that the neurons in the transplanted claw terminate largely within the host's ipsilat-

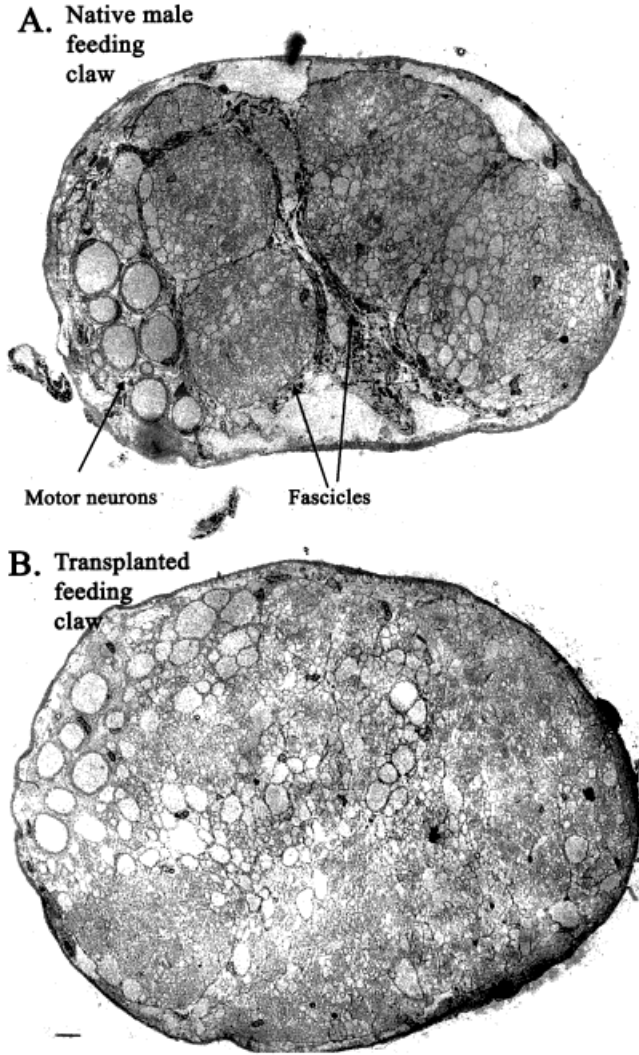


Fig. 2. Transmission electron micrographs of a transverse section of the second nerve root from native and transplanted feeding claws of a single male *U. pugilator* receiving a feeding claw from a female donor. Roots are oriented so that medial is toward the top, lateral to the bottom, dorsal to the right, and ventral to the left. Both images $\times 620$. **A:** Nerve root from the male feeding (native) claw. **B:** Nerve root from transplanted feeding claw from a female donor. Scale bar = 100 μm .

eral, rather than contralateral, hemiganglion. We could discern no evidence in any of the three preparations that axons continued to grow through the ipsilateral neuropil, which is a region of the CNS normally free from chemosensory input in adults, to innervate the contralateral hemiganglion that normally processes chemosensory input. Although we cannot exclude the possibility that greater incubation time may have revealed neurons that do indeed cross the midline, stained nerves seemed to fill the entire ganglion, suggesting fairly complete penetration. DiI fills also revealed that the first thoracic hemiganglia were similar in size. The lack of distinct hypertrophy of the hemiganglion formerly associated with the major claw is again in contrast to the patterns in normal male fiddler crabs, in which the volume of the hemiganglion

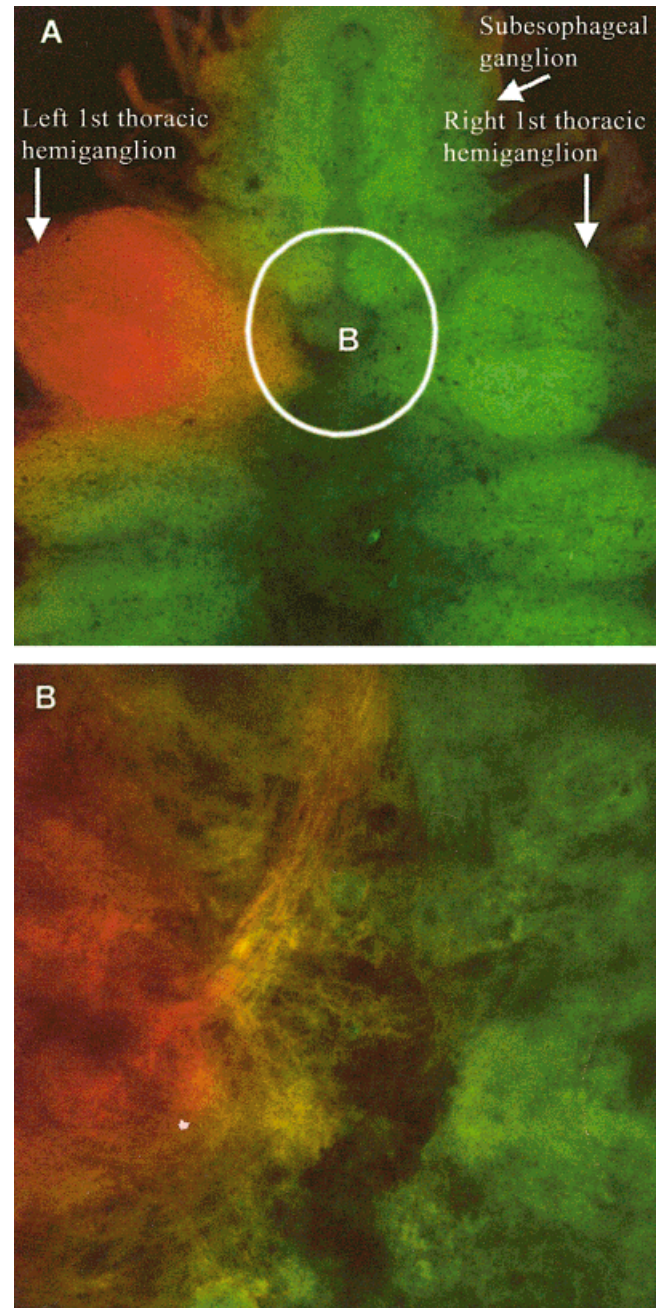


Fig. 3. Confocal micrographs of the fused thoracic ganglion of a male *Uca pugilator* with a successfully transplanted feeding claw from a female donor. **A:** Lower power image. The subesophageal ganglion is at the top, followed sequentially by five bilateral pairs of hemiganglia for each of the thoracic segments. The figure shows DiI fluorescence (red) from the afferents of the transplanted claw entering the right first thoracic hemiganglion, which formerly received input from the male major claw. The sensory axons from the transplanted claw appear to terminate in the ipsilateral hemiganglion and do not cross the midline to the contralateral hemiganglion, which receives the chemosensory input from the native feeding claw. $\times 5$. **B:** Higher power image of the medial one-third of the hemiganglion. Note the cell clusters near the medial edge. $\times 20$.

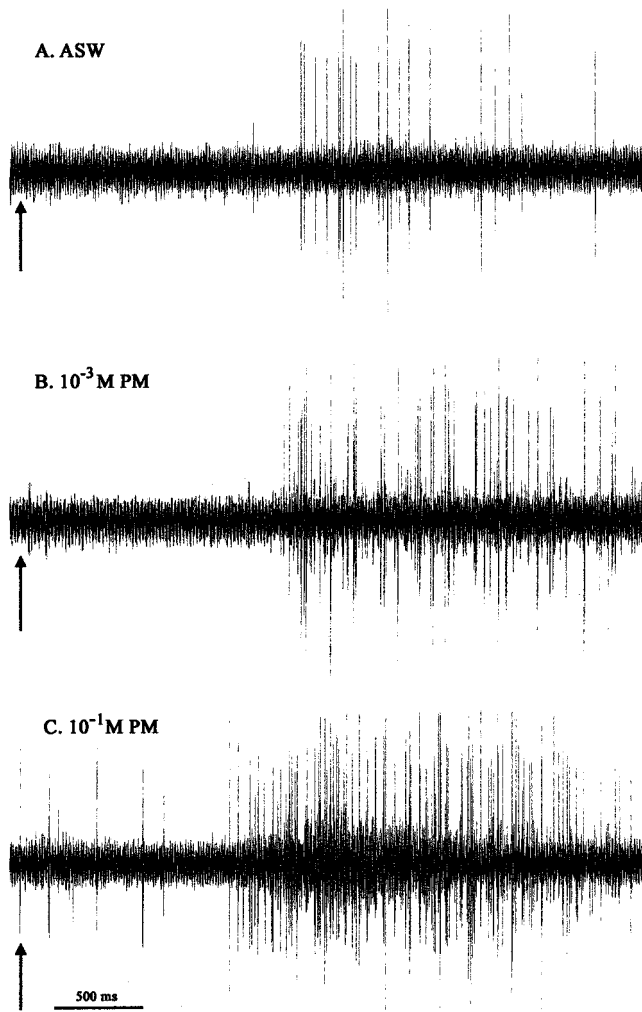


Fig. 4. Typical spike trains from neurons in transplanted claws in response to plant mix and ASW. Each panel shows the response when neurons in the claw were challenged with a particular stimulus. The traces begin with the introduction of the test stimulant, denoted by the arrow. The responses are to ASW (A), 10^{-3} M plant mix (B), and 10^{-1} M plant mix (C). The traces are from a single experiment and show the activity of more than one neuron.

receiving inputs from the major claw is considerably greater than that of the ganglion associated with the feeding claw (Young and Govind, 1983).

Physiology of chemosensory neurons

The morphological characterization of claw type was validated using physiological tests, indicating that claws designated as feeding claws derived from either male or female donors possessed chemosensory abilities. Neurons in successfully transplanted claws respond to chemical stimulants (Figs. 4, 5), whereas claws of transplant controls, claws designated as failed transplants, or regenerating major claws never exhibited chemosensory responses (Weissburg, unpublished observations). We could discern no obvious difference in spike amplitude or other waveform characteristics of neurons in successfully transplanted claws relative to responses of chemosensory neurons from normal male or female feeding claws.

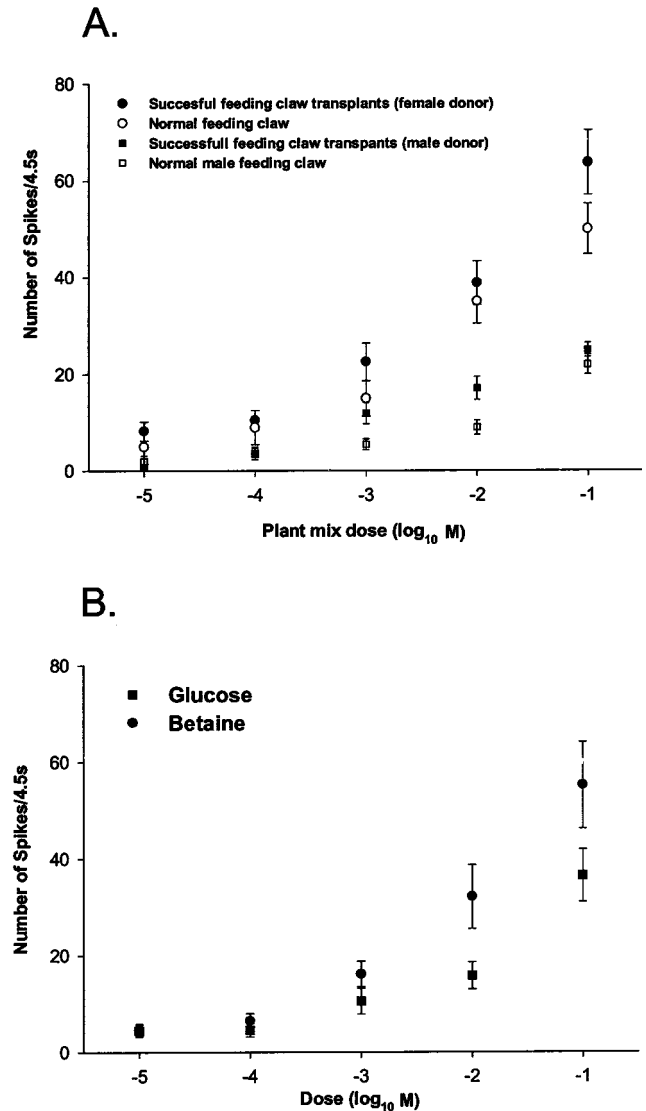


Fig. 5. Dose-response function of chemosensory neurons in successful feeding claw transplants from male and female donors. **A:** Mean \pm standard error for responses of 19, 13, 10, and 11 chemosensory neurons from successfully transplanted feeding claws from female and male donors and from feeding claws from normal females and males, respectively, in response to plant mix. Data from normal males and females are from Weissburg and Derby (1995). **B:** Mean \pm standard error for responses to betaine and D-glucose from 12 chemosensory neurons from successfully transplanted female feeding claws. Some animals died prior to testing and yielded only morphological measurements, so the number of physiological tests is less than the number of successful surgeries listed in Table 1.

The dose-response functions of neurons are characteristic of the sensitivities of the donor limb type (Fig. 5). There is a slight, statistically significant increase in the sensitivity of neurons from transplanted claws relative to neurons from normal individuals of the same sex; neurons from transplanted male feeding claws are slightly (~ 10 – 30%) more sensitive to PM than are neurons from non-transplanted male feeding claws, with a similar effect seen in transplanted female vs. native female feeding

claws ($F_{1,170} = 15.4$, $F_{1,194} = 11.3$ for males and females, respectively; $P < 0.01$ in both cases). However, in spite of the general increase in sensitivity resulting from transplantation, the sex-specific difference in sensitivity normally seen in chemosensory neurons of fiddler crabs is preserved. The dose-response functions of neurons from female vs. male transplanted feeding claws are significantly different ($F_{1,167} = 96.8$; $P < 0.001$). In fact, because the magnitude of the increase in the sensitivity of transplanted claws is nearly equal in claws from male and female donors, the proportionate increase in sensitivity of neurons in transplants is almost exactly the same as that normally observed for neurons in the claws of intact animals. At a concentration of 10^{-4} M, just above threshold for males, there is a 2.6-fold increase in sensitivity of normal female vs. male neurons and also in neurons from transplanted female vs. male claws. At 10^{-1} M, the sensitivity in female neurons increases by 2.3-fold and 2.4-fold for native and transplanted claws, respectively. There is little evidence of heightened variation in sensitivity of neurons in transplanted female feeding claws relative to that expressed by neurons in normal females or males. This argues against the increased sensitivity of neurons in the transplants being the result of a subpopulation of cells with particularly exquisite sensitivity. Rather, as we have previously documented in normal animals (Weissburg and Derby, 1995; Weissburg, 1999), it appears that, on average, the sensitivity of neurons in female-derived transplants is greater than that of the male's native claw.

The responses of neurons in transplanted female claws to a very limited set of single compounds (Fig. 5B) are consistent with sensitivities seen in normal female animals (Weissburg, 1999). Response magnitudes are characteristic of those recorded from neurons in females, although the high variability of sensitivity to single compounds makes this pattern less clear than in the case of PM. Betaine appears to be a more efficacious stimulant than D-glucose, which parallels our previous observations on the ranking of these two chemicals when presented to neurons in the feeding claws of normal animals (Weissburg, 1999). These observations suggest that the neurons of successfully transplanted feeding claws continue to express the normal suite of sex-specific properties. In particular, neurons from female claws retain their characteristic properties even when transplanted onto a male host and function in a similar manner in transplanted claws compared to their behavior in normal, unmanipulated female claws.

Behavior

Although the physiological experiments indicate that chemosensory neurons in successfully transplanted feeding claws respond to chemical stimulants, these experiments leave open the question regarding the functional role of transplanted feeding appendages in mediating behavior. Behavioral experiments with crabs bearing successfully transplanted claws were necessary to determine whether these claws mediated feeding and whether behavioral sensitivity reflected the differences in sensitivity of the transplanted feeding claw.

Strikingly, the transplanted claw mediates feeding activity in response to chemical cues. Focal application of chemical stimulants to both native and successfully transplanted feeding claws evoked feeding behavior in a dose-dependent fashion (Fig. 6). Mechanical stimulation ap-

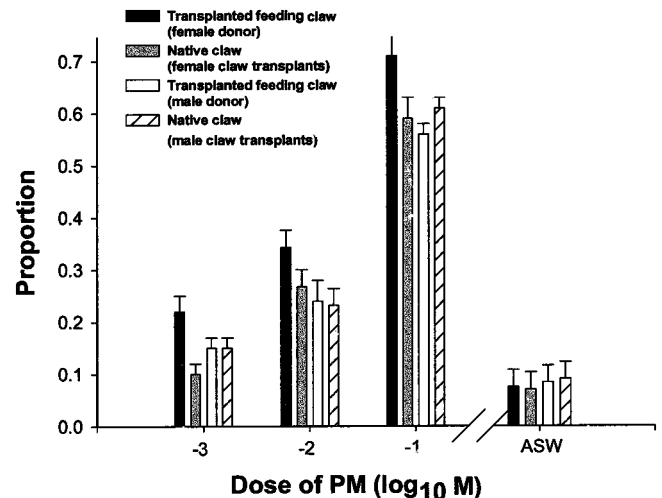


Fig. 6. Behavioral response of males with feeding claw transplants from male and female donors. Proportion of time for which stimulation of the claw with PM and ASW elicited feeding. Data represents the mean proportion of stimulations, \pm stdev, that resulted in feeding motions in an individual crab with a given claw type, averaged across all crabs with that claw type. Transplanted feeding claws are identified as originating from either a female or a male donor. *Native claw* refers to the feeding claw originally present on the male, with the origin of the male's transplanted claw given in parentheses. Sample sizes were 40 and 29 for successful transplants from female and male donors, respectively. Some animals died prior to testing and yielded only morphological measurements, so the number of behavioral tests is less than the number of successful surgeries listed in Table 1.

pears to evoke a low frequency of feeding motions for all cases, as evinced by the response to ASW. However, chemical stimulation of claws designated as failed transplants, claws of transplant controls, or regenerating major claws never evoked feeding activity greater than that produced by ASW, indicating they are unresponsive to chemical stimuli (data not shown). The sensitivity of the animal's native claw appeared similar across the two transplant groups (Fig. 6), as well as being similar to that of native claws from animals designated as failed transplants (data not shown), indicating that our protocol resulted in an accurate, if crude, assessment of behavioral chemosensitivity.

Transplanted claws evoked more frequent feeding when presented with higher concentrations of PM. The effect of dose was significant for animals receiving transplants of both male and female feeding claws ($\chi^2 > 288$, $df = 3$, $P < 0.001$ for both cases). However, there was a perceptible difference in the behavioral sensitivity mediated by transplanted claws from female vs. male donors. Although the paired test design made it statistically impossible to compare the sensitivity of the two types of transplanted claws directly, we did compare the behavioral sensitivity on the native vs. transplanted claws for each of our two experimental groups of successfully transplanted claws. We excluded animals bearing unsuccessfully transplanted (e.g., major) claws, given the obvious lack of response to any stimulus from males in the failed transplant group.

The analysis revealed that, in animals receiving claws from female donors, stimulating the transplanted claw resulted in significantly greater feeding activity than that produced by the male's native feeding claw ($\chi^2 = 7.99$, $df =$

1, $P < 0.05$). Corrected for response to the ASW controls, the magnitude of this increase ranged from nearly three-fold at 10^{-3} M to 1.20-fold at 10^{-1} M. Individual comparisons indicated that the transsexually grafted feeding claws evoked significantly greater feeding activity at all concentrations of PM (maximum likelihood $G > 6.1$, $df = 1$, $P < 0.05$, for all comparisons), but not when ASW was the stimulus ($G = 4.9$, $df = 1$, $P > 0.05$). In contrast, we could detect no increase in behavioral sensitivity for animals receiving transplants of male feeding claws compared to the sensitivity of the male's native side ($\chi^2 = 0.28$, $df = 1$, $P > 0.05$). That is, the transplantation of a female claw specifically and significantly increased the sensitivity of the behavioral response to chemical stimulants relative to the sensitivity evoked by the male's native feeding claw.

DISCUSSION

Our experiments indicate that the natural regenerative capacities of crustaceans result in the ability to equip a male crab with additional chemosensory feeding appendages, rather than their normal complement of one. Developing limb buds from either male or female donors can be successfully grafted to males in place of their major claws, to equip males with a second chemosensory feeding claw (Fig. 1). Basic patterns of neuroanatomy, including the lack of bilateral asymmetry in the size of the first thoracic ganglion (Figs. 2, 3), also are consistent with the properties of the feeding claw, rather than that of the major claw that formerly occupied the transplant site. The transplanted feeding claw appears to maintain its physiological competence and sex-specific properties (Figs. 4, 5). This indicates that transplanted limbs seem to be largely autonomous and to legislate their own development even in a foreign developmental context. Ingrowing chemosensory axons from transplanted feeding claws make functional connections in the CNS normally devoted to processing nonchemosensory input emanating from the male's major claw and, instead, evoke feeding behavior (Fig. 6). Thus, even though the transplanted neurons project to a neuropil that does not appear to process chemosensory input, their interactions within this foreign neuropil are sufficient to drive the appropriate behavior. In fact, sex-specific sensitivities of neurons in the transplanted feeding claw translate into greater behavioral sensitivity of males receiving transsexual limb transplants than that of males receiving transplants from a male donor (Fig. 6).

Reorganization underlying transplantation

Several transplant studies have suggested that neurons transplanted to foreign locations successfully navigate to the CNS, presumably using molecular markers to find their way. Proprio- and tactile receptors can be induced to project to their segmentally homologous neuropil when transplanted ectopically to a foreign location (Killian et al., 1993). In moths receiving transsexual antennal grafts, sensory axons maintain their sex-specific sensory projections and form sex-specific olfactory glomeruli in the same position that they occupy in normal animals (Rössler et al., 1999). These and other experiments in vertebrates and invertebrates (see, e.g., Sur et al., 1990; Passani et al., 1991) suggest that positional cues are often available, even if not specifically needed, to mediate pathfinding of a particular group of axons during normal development.

Similarly, ingrowing chemosensory axons from transplanted claws in fiddler crabs appear to experience appropriate molecular markers and terminate in the ipsilateral ganglion even though this area of neuropil does not normally accommodate this modality.

Once within this ganglion, chemosensory axons establish connections sufficient to drive appropriate chemosensory behavior. Ectopically transplanted neurons often maintain largely normal projection patterns in foreign neuropil (Murphey et al., 1983; Edwards, 1988). The fidelity with which neurons arborize in particular spatial regions of the ganglion allows them to form synapses with neurons with which they normally associate even though these connections are established in an unfamiliar neural substrate (Killian et al., 1993). Occasionally, transplanted neurons form novel connections with different partners that have dendrites in the same region where the transplanted neurons arborize but which are not found in the neuropil normally occupied by the transplants (Murphey et al., 1983). This suggests that particular patterns of connectivity that underlie specific neural functioning may occur even when neurons project to a novel region of the CNS, although the behavioral consequences of these connections have been largely unexplored. Clearly, transplanted chemosensory axons in fiddler crabs are able to form functional connections sufficient to produce normal behavior even when induced to grow into a different hemiganglion.

We interpret the behavioral experiments as also suggesting that novel sensory capabilities may be conferred on CNS elements when innervated by sensory neurons not normally associated with a given neuropil. In fiddler crabs, receptor-rich feeding claws are able to drive feeding behavior when transplanted to the site of mechanosensory male major claws. Cross-modal connections have been produced in other systems, indicating that sensory neurons may utilize novel targets when deprived of their normal postsynaptic partners (Sur et al., 1990; Scalia et al., 1995; Yaka et al., 1999). However, to our knowledge, this is the first demonstration that CNS elements with rewired connections can successfully process sensory stimuli resulting in behavioral discrimination of stimulus intensity.

The supposition that our transplant experiments represent cross-modal reorganization is based on strong evidence that the hemiganglion receiving transplanted chemosensory axons does not participate in chemosensory processing. This is certainly the case in males over the majority of their lives. A variety of evidence also suggests that, in spite of the fact that the claw dimorphism is inducible, the default condition of this neuropil is to process input from the nonchemosensory major claw in adults. Prior to the development of the claw asymmetry in males, which occurs in 1–2 mm animals recently molted from their oceanic larval phase, both claws are major. Damage to one of the claws during this stage sets the asymmetry; the damaged claw develops into a feeding claw on the subsequent molt (Morgan, 1924; Yamaguchi, 1977).

Indeed, we very rarely have seen small juveniles that have fortuitously escaped damage to either claw and consequently sport two major claws rather than the traditional dimorphic arrangement. In such animals, neither of their major claws appears to be chemosensory based on physiological or behavioral assays (Weissburg, unpub-

lished observations). These males probably use chemoreceptors on the tips of their walking legs (Weissburg and Derby, 1995) to sense chemical cues. Even if the hemiganglia innervated by the major claw of adults possess relics associated with chemosensory input from prior life stages, reorganization of the thoracic ganglion by ingrowing chemosensory axons from the transplanted feeding claw would be necessary.

The reduction in size of hemiganglia associated with transplanted feeding claws, but that previously received input from major claws, is the most immediate consequence of the changes in the neuronal properties resulting from limb transplantation. There are other potential changes as well, which are associated with a variety of differences in the organization of these two claw types. For instance, major and feeding claws differ significantly in the number of axons and the size-frequency distribution of their axonal population impinging on the CNS. Axons with diameters greater than 1 μm make up approximately 69% vs. 85% of the population projecting to the major vs. feeding claw hemiganglia, respectively (Young and Govind, 1983; Weissburg et al., 1996). The diameters of motor neuron axons and somata are 30–60% larger in major than in feeding claws (Young and Govind, 1983). Finally, the modality-specific patterns of organization repeatedly observed in many other animals argue that ingrowing chemosensory axons would arborize in regions different from those of the mechanosensors that normally carry sensory information into the major claw hemiganglion.

Peripheral vs. central effects in the production of sex-specific behavior

Successfully transplanted feeding claws not only are morphologically indistinguishable from normal feeding claws but contain sensory neurons that remain faithful to their donor origin, even when males receive transsexual limb grafts. In other invertebrates, individual sensory neurons (Possidente and Murphey, 1989; Passani et al., 1991) or muscle types (Trinkaus-Randall and Mittenthal, 1978) that are sexually dimorphic retain their properties in transplantation experiments, although experimental production of sexually dimorphic appendages appears rare. The physiological experiments showing that sensory limbs maintain the sensitivity characteristic of the donor are consistent with the greater behavioral sensitivity expressed by males receiving feeding claws from female, but not male, donors. Using a common CNS substrate, the more sensitive chemoreceptors on the transsexually grafted feeding claw evoked more frequent feeding behavior. This implies that, regardless of any differences in CNS elements between males and females, changes in the properties of the periphery may directly modulate quantitative sex-specific differences in behavioral chemosensitivity.

The ability of chemosensory systems to regain function following tissue transplantation appears robust, in that experiments in both vertebrates (Hendricks et al., 1994; Goheen et al., 1995) and invertebrates (Schneiderman et al., 1986; Murphey et al., 1983; Rössler et al., 1999) show that chemosensory neurons reestablish connections in olfactory neuropil following transplantation and mediate olfactory-induced behaviors. The attraction of male moths to female sex attractants has been successfully recapitulated in females by transplantation of male antennal disks into developing females (Schneiderman et al., 1986; Willis et al., 1995). This is similar to our results; both fiddler

crabs and moths show induction of sex specificity via transplantation of peripheral chemosensory structures. However, unlike the case in fiddler crabs, neurons in both native and transplanted antennae of moths project into a chemosensory neuropil, whereas neurons in the transplanted claw of fiddler crabs terminate in a sexually dimorphic region of the CNS that does not receive chemical input in normal (adult) crabs prior to the limb graft. The extent to which this sex specificity relates to the intrinsic properties of the sensory neurons, rather than potential sex-specific patterns of organization in the CNS as a result of interactions with these sensory neurons, remains to be explored.

Neural plasticity and sensory processing

Neuronal guidance and target selection are presumed to reflect mechanisms necessary to ensure that precise and stereotyped synaptic connections are established, creating the neural architecture required for normal operation of the CNS. The chemosensory axons in transplanted feeding claws of fiddler crabs may be able to reorganize local circuits in the neuropil that normally receive input from the major claw, such that the ability to process chemosensory signals emerges. Such plasticity has been suggested for other systems (Sur et al., 1990; Yaka et al., 1999). However, it has also been argued that processing deficits arise from patterns of neural architecture that are insufficient for particular tasks but cannot be modified by invasion of novel sensory afferents (Scalia et al., 1995).

Alternatively, such a precise pattern of connections may be unnecessary for the relatively simple process of intensity discrimination, and existing circuitry may be sufficient. Experiments in moths have suggested that the normal glomerular organization of olfactory neuropil is not necessary for upwind flight in response to odor plumes and that this motor program can be triggered in moths of reduced anatomical complexity (Willis et al., 1995). Rats recover olfactory ability following transplantation, although they are not as proficient at olfactory location of items as are normal animals, perhaps because transplantation does not result in completely natural patterns of connectivity and levels of glomerularization (Goheen et al., 1995; Evers et al., 1996). We are eager to see whether the typical pattern of neuropil organization is maintained in fiddler crabs receiving transplanted appendages and whether this impacts on more challenging tasks, such as discrimination of odor qualities.

ACKNOWLEDGMENTS

We thank H.S. Cate and M. Mulkeen for their technical assistance at various phases of this study, P.J.H. Harrison for confocal microscopy, and C.K. Govind for electron microscopy. Comments by P. Steullet, S. Cromarty, and S. Pallas greatly improved this work. We thank J. Thompson for his prodigious memory and P.M. Hopkins for advice on the induction of molting. Two anonymous reviewers provided comments that helped to improve the text. These studies were supported by NIH grants to M.J.W. (DC02731) and C.D.D. (DC00312).

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