Regeneration of Cercal Filiform Hair Sensory Neurons in the First-Instar Cockroach Restores Escape Behavior

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ABSTRACT: **Neural regeneration in the escape circuit of the first-instar cockroach is described using behavioral analysis, electrophysiology, intracellular staining, and electron microscopy. Each of the two filiform hairs on each of the animal's cerci is innervated by a single sensory neuron, which specifically synapses with a set of giant interneurons (GIs) in the terminal ganglion. These trigger a directed escape run. Severing the sensory axons causes them to degenerate and perturbs escape behavior, which is restored to near normal after 4–6 days. Within this time, afferents regenerate and reestablish arborizations in the terminal ganglion. In most cases, regenerating afferents enter the cercal glomerulus and re-form most of the specific monosynaptic connections they acquired during embryogenesis, although their morphology deviates markedly from normal; these animals reestablish near normal escape behavior. In a few cases, regenerating afferents remain within the cercus or bypass the cercal glomerulus, and thereby fail to re-form synapses with GIs; these animals continue to exhibit perturbed escape behavior. We conclude that in most cases, specific synapses are reestablished and appropriate escape behavior is restored. This regeneration system therefore provides a tractable model for the establishment of synaptic specificity in a simple neuronal circuit.** *J Neurobiol* 33: 439–458, 1997

Keywords: **development; escape behavior; *Periplaneta americana*; synapse formation; regeneration**

INTRODUCTION

Escape circuits in vertebrates and invertebrates offer tractable material for neuroscientists. The neurons in these circuits are often large and accessible, the circuits are relatively simple, and the escape behaviors they mediate are often stereotyped and amenable to analysis.

The first-instar cockroach escape behavior circuit offers a very accessible set of neurons for analysis, owing in particular to their visibility with Nomarski optics (Blagburn, 1989; Bacon and Blagburn, 1992). The animal has a pair of cerci at its rear end, each of which bears two wind-sensitive hairs, lateral and medial. Each hair is innervated by a single sensory neuron; these four afferents project to the terminal ganglion, where they synapse with a set of identified giant interneurons (GIs) (Blagburn,

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1989). The GIs ascend to the thoracic ganglia, where they synapse with interneurons which are presynaptic to leg motoneurons, thus completing the escape circuit (Ritzmann, 1981). Because the sensory afferents innervate hairs which respond to different wind directions (Dagan and Volman, 1982), the central nervous system (CNS) is able to compute the direction of an air disturbance and direct the orientation of the escape response accordingly. The accuracy of the escape behavior relies on the precision of synaptic connectivity as this neural circuit is established during embryogenesis.

Our work focuses on the developmental mechanisms that lead to specificity in a subset of these synaptic connections: the sensory neuron (SN) to GI synapses. The normal embryonic development of some of these synapses has recently been described using a combination of physiologic and anatomic techniques (Blagburn et al., 1996), but to understand these developmental mechanisms further, surgical (e.g., specific cell ablation) and molecular (e.g., perturbation of gene expression) manipulations will be required. However, the embryo is not well suited for most of these manipulations because of its fragility, the small size of neurons, and the difficulty of embryo culture for prolonged periods. A regeneration paradigm for the first instar would circumvent many of these problems and would also offer the advantage of enabling us to test the outcome of these manipulations behaviorally. Regeneration of neurons has been studied in a number of invertebrate and vertebrate systems (Nicholls, 1987; Sah and Frank, 1984; Chiba and Murphey, 1991; Noel et al., 1995) and is often assumed to represent a good model for embryonic development of those neurons. There are few preparations, however, in which this assumption has actually been tested. The cockroach preparation described here allows us to directly compare the accuracy of regeneration with that of embryonic development.

In the present study, we forced the regeneration of afferent neurons by crushing one of the cerci of a newly emerged first-instar cockroach. We addressed two main questions: (a) Do regenerating filiform hair afferents attain their original morphology and establish connections with the same accuracy as during embryogenesis? and (b) Is this neural regeneration necessary and sufficient to restore the original accuracy of the escape behavior after crushing the afferents?

We show that behavior recovers at the same time as regenerating neurons start to re-form appropriate synaptic connections. However, escape behavior remains perturbed in those few individuals in which severed afferents fail to regenerate into the cercal glomerulus. In the majority of individuals, sensory afferents regenerate into the cercal glomerulus and, despite some morphologic differences to the original afferents, reestablish their specific connections with a high degree of precision.

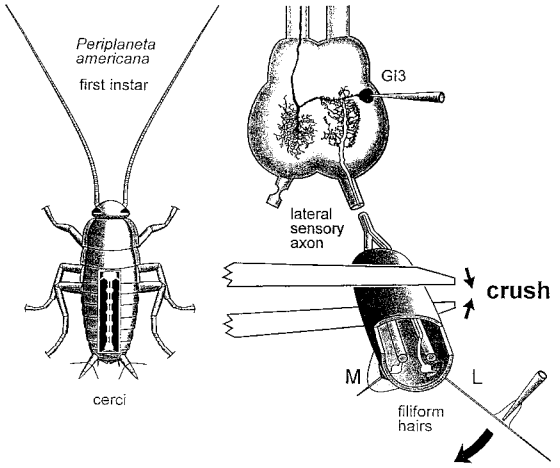


Figure 1 Diagrammatic representation of the first-instar cercal system. The sensory afferents L and M synapse with GIs in the terminal ganglion (only L is shown with ipsilateral GI3). Crushing the cercus at its base with forceps severs the axons of L and M.

MATERIALS AND METHODS

Animals

First-instar wild-type cockroaches (*Periplaneta americana*) from our laboratory cultures were used in all experiments.

Sensory Nerve Crush

One day after hatching, cockroaches were immobilized by chilling them over crushed ice for at least 10 min. The base of the proximal segment of the right cercus was crushed carefully using a pair of fine forceps (Fig. 1). To identify individual animals, white enamel paint was applied to the animals' tergites in unique patterns using a sharpened toothpick.

Behavior

Experimental Setup. Behavioral experiments were carried out in a Perspex arena (diameter 14 cm, height 4 cm) with a roughened plastic floor. Three Perspex cloverleaf inserts were fixed to the inside of the arena at 90° to each other; this encouraged the highly thigmotactic animals to explore the center of the arena. A wind stimulus device

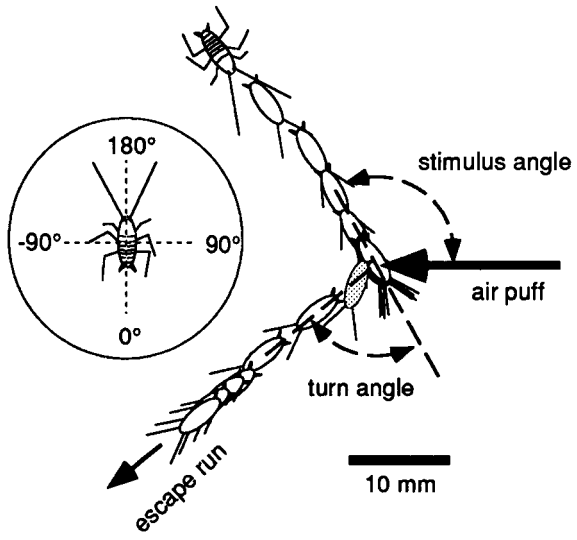


Figure 2 Schematic track of an escape run of a first-instar cockroach as revealed by single-frame video analysis. The datum resulting from this escape run is represented as a cross in Figure 3(A); the measured stimulus angle is −112°, the turn angle is 76°. The sample interval was 40 ms except for the stippled outline (20 ms). The insert indicates the directions as used in the text.

capable of delivering standard air puffs within the arena was constructed as outlined by Kolton and Camhi (1995). In our case, the loudspeaker was used to drive wind through a wind tube positioned at 45° from the vertical and 7 mm from the floor. To identify the time of stimulus onset, a small light-emitting diode was coupled to the stimulus generator. A hot-wire anemometer was used to calibrate the wind velocity within the circular target zone (diameter 2.5 cm). Wind velocity ranged from 0.7 to 0.8 m/s. Visualization of the wind stream in a plume of smoke confirmed that the stimulus was laminar within the target zone.

Measurement and Evaluation. Behavioral responses were filmed from above using a black-and-white Sony CCD video camera (SSC-M350CE) at 50 frames/s. Some animals were tested on each postcrush day, from day 1 (PC1) to day 6 (PC6); others were tested only on PC1 and PC6. Prior to testing, animals were cooled on ice to facilitate their inspection; animals with damaged cercal or other appendages were removed from the experiment. Animals were placed in the arena in groups of five and allowed to acclimatize for 30 min. They were tested in trial sessions of 10 min each with a 30-min rest period between sessions. Quiescence was not considered a response. The angle of escape and angle of wind presentation were measured as defined by Camhi and Tom (1978) using a custom-made analysis program; screen distortion was rectified via calibrations built into the program. The angle of turn was defined as the angular difference between the animal's orientation before and after the escape turn (Fig. 2). Turns to the right were classified as positive and turns to the left as negative. The animal's rostral end was designated as 180° and its caudal end as 0° (Fig. 2).

Dissection

Central nervous system and cerci were isolated according to the method of Blagburn (1989). Briefly, animals were cold-anesthetized over crushed ice and placed in saline. Appendages and most of the abdominal cuticle, fat body, and gut were removed. The preparation was anchored upside down in a silicone-walled chamber built on a glass microscope slide, using petroleum jelly. The sheath of the terminal ganglion was removed using finely sharpened forceps. The slide was then transferred to the stage of an upright microscope equipped with Nomarski optics and a ×40 water-immersion lens. For impalement of sensory neuron cell bodies, the distal two cercal segments were removed and the dorsal cuticle of the proximal segment was cut off with sharpened iridectomy scissors (Fig. 1). Either the L or M sensory neuron was stimulated by moving its hair using a Vaseline-coated broken micropipette mounted on a loudspeaker that was connected to a computer-controlled pulse generator. The other sensory hair was immobilized by covering its base with Vaseline (Fig. 1). The left (untreated) cercal nerve was crushed before the experiment to remove input from this side.

Electrophysiologic Recording

Micropipettes were filled with 2 *M* potassium acetate to give resistances between 30 and 60 MΩ. The cell bodies of GIs and, in a few cases, sensory neuron axons were identified using Nomarski optics by their size, position, and overall appearance as described elsewhere (Blagburn, 1989). Recordings were rejected if the membrane potential failed to stabilize at or below −70 mV. GI7 was excluded from the investigation, because it only rarely receives monosynaptic filiform hair input (Blagburn, 1989). The terms ''ipsilateral'' and ''contralateral'' refer to the position of the GI cell bodies.

Staining of Afferents

Cell bodies of sensory neurons were impaled with electrodes (40–100 MΩ) containing 4% Lucifer yellow (Molecular Probes, Eugene, OR) in the tip, backfilled with 2 *M* LiCl. Cells were dye-filled by application of either −0.5 to −1 nA DC current, or 200 ms hyperpolarizing pulses of 1–2 nA at 2 Hz until the cell body appeared yellow (usually after 3–20 min). After filling, the preparation was left at room temperature for 15 min to allow for dye diffusion and then fixed for 1 h in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Preparations were washed in PBS, dehydrated in a graded alcohol series, cleared in methylsalicylate, and viewed under epifluorescence. The preparations were then rehydrated and processed using an anti-Lucifer yellow anti-

body (Molecular Probes, Eugene, OR) and the ABC method (Vector, Burlingame, CA). Preparations were mounted in Canada Balsam and drawn using a drawing tube.

Electron Microscopy

Neurons were injected with horseradish peroxidase (HRP, Sigma Type VI). The tips of the microelectrodes were filled with a 4% solution of HRP in 0.2 *M* Tris buffer with 0.3 *M* KCl, pH 7.0, and the shank was backfilled with 1 *M* KCl. After impalement of the neuron, depolarizing current pulses 2–4 nA in amplitude and 0.5 s in duration were passed through the microelectrode at a frequency of 1 Hz for periods of approximately 6 min. After injection, the ganglia were left in aerated saline for 10 min to allow for transport of the enzyme. The tissue was then fixed at 4°C for 1–2 h in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer with 0.5 *M* sucrose, pH 7.4, washed for several hours in the same buffer, transferred to 0.1 *M* Tris buffer, pH 7.4 for 10 min, then placed in a 0.5% solution of cobalt chloride in Tris buffer for 10 min to intensify the HRP reaction product. After a 10-min wash in Tris buffer, the ganglia were transferred to a reaction medium containing 5 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Isopac), 30 μL glucose oxidase (Sigma, Type V), and 20 mg β-D-glucose in 10 mL phosphate buffer, adjusted to pH 7.2. After 10–20 min incubation in this medium in the dark at 30°C, the tissue was washed in cold cacodylate buffer, then postfixed in 1% osmium tetroxide for 1 h. After washing in buffer for 10 min, the ganglia were stained with 3% uranyl acetate in 70% ethanol for 30 min, dehydrated in ethanol, and embedded in Araldite/Medcast. Thin sections (60 nm) were collected on copper hexagonal thin bar grids, then counterstained with uranyl acetate and Reynold's lead citrate. Grids were examined with a Philips EM 401 electron microscope.

Statistics

Throughout the report, values are given as mean ± standard error of the mean. For comparison of scattergrams of directional escape responses, a Monte Carlo randomization algorithm was used (Manley, 1991; Zar, 1975).

RESULTS

Behavior

The Escape Behavior of the First-Instar Cockroach Is Highly Stereotypic. When presented with a wind stimulus, first-instar cockroaches turned away from the stimulus and subsequently executed an escape run (Fig. 2). The theoretically ideal behavior is indicated as a dotted line in Figure 3(A); the majority of the animals turned somewhat less. Winds from the right evoked left turns; winds from the left evoked right turns. The experiments described here relied on our being able to analyze the escape behavior of individually identifiable animals for up to 6 consecutive days after the crush. To identify individual animals, unique patterns of white paint were applied. In addition, each day, the animals were cooled down on ice to check their condition prior to testing; the behavior of 25 individually marked control animals that were treated in this way [Fig. 3(A)] did not differ from the normal behavior of untreated animals (Dagan and Volman, 1982) (Ediger, unpublished results).

Escape Behavior Is Perturbed 1 Day after Cercal Crush. We analyzed the escape behavior of that subset of crush treated animals that could be sampled with wind from the right a minimum of five times on day 1 postcrush (PC1) and then subsequently a minimum of five times on day 6 postcrush (PC6). The escape behavior of these 20 animals 1 day after the right cercus of each had been crushed [Fig. 3(B)] differed significantly ($p < 0.0001$; Monte Carlo randomization test, 10,000 permutations) from that of the individually marked controls [Fig. 3(A)]. Unless stated otherwise, all subsequent statistical tests are Monte Carlo. By restricting our statistical analysis either to responses to wind from the right (crushed) or the left (uncrushed) side, we found that only the responses to wind from the treated side were affected: the response of treated animals [Fig. 3(B)] to stimuli from the right (0° to +180°) differed significantly from that of individually marked controls [Fig. 3(A)] ($p < 0.0001$), with treated animals turning more frequently toward the wind stimulus. In response to wind stimuli from the left (−180° to 0°), the evasive behavior of treated and control animals did not differ significantly ($p = 0.090$).

An alternative way of analyzing individual responses is to classify turns away from the wind as correct and turns into the wind as incorrect (Camhi et al., 1978). Although this classification is somewhat arbitrary and oversimplifies the accuracy of escape behavior, it is useful for determining whether the animal's ability to differentiate between right and left winds was disturbed by treatment. For each animal, the percentage of correct responses to wind from the right was determined. The mean percent correct responses for the population of 20 animals on PC1 (39.7 ± 4.7%) was significantly lower ($p < 0.0001$, t test) than that of 15 individually marked control animals (89.3 ± 3.9%) [Fig. 3(D)]. This

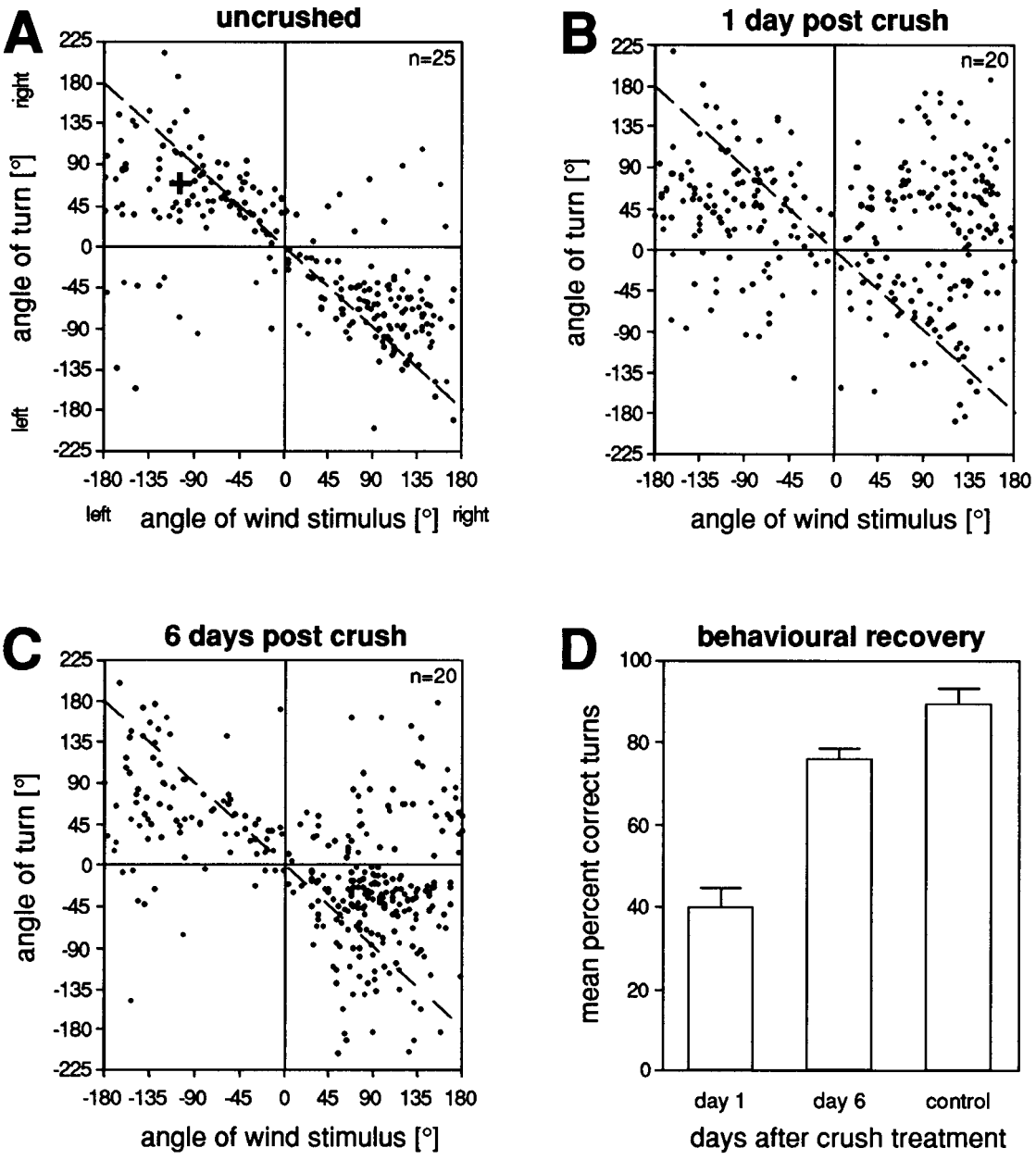


Figure 3 Escape behavior returned to near normal 6 days after crush treatment. (A) Escape behavior demonstrated by 25 individually marked control animals. The dashed line ($y = -x$) in (A–C) indicates the theoretically ideal escape behavior. The escape turn shown in Figure 2 is indicated by a cross. (B) One day after crushing the right cercus, responses to right winds usually resulted in (inappropriate) turns into the wind. Each of the treated animals ($n = 20$) was tested with right wind a minimum of five times on both PC1 and PC6. (C) By 6 days after treatment, these same 20 animals executed fewer incorrect turns in response to right wind. (D) Turn accuracy of treated animals, to right winds, increased by day 6. The percent correct responses for each of the 20 individual animals (B,C) were averaged on PC1 and PC6. Control data represent responses of the subset of individuals ($n = 15$) in (A) that was sampled at least five times with right wind.

result could be due to incomplete crushes in some individuals. In this case, the population would consist of two groups of animals, one of which had been successfully treated and therefore always turned incorrectly, and the other group treated unsuccessfully, leaving escape behavior normal. How-

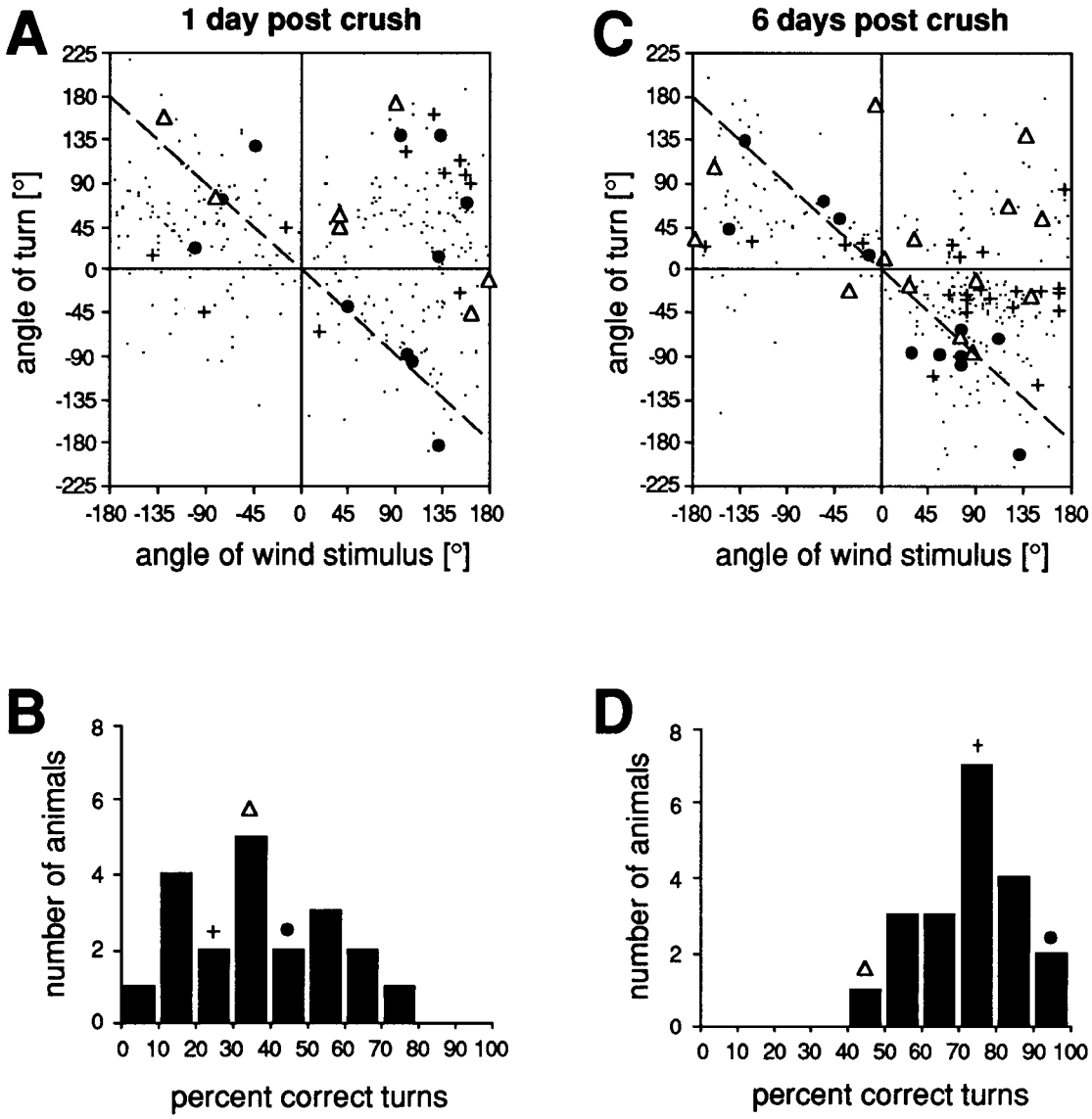


Figure 4 Most animals recovered their escape behavior; a minority did not. (A) At PC1, escape behavior was perturbed [same data as in Fig. 3(B)]. The responses of three individuals are identified by different symbols (triangles, crosses, and circles). (B) Histogram showing the distribution of turn accuracy in response to right wind on PC1 within the group of 20 animals shown in (A). Symbols indicate the positions of the three highlighted individuals in (A) in the histogram. (C) Turn accuracy improved by PC6 but varied between individuals. The same data as Figure 3(C), but the responses of the same individuals as in (A) identified by the same symbols as in (B). (D) Histogram showing the distribution of turn accuracy on PC6 with symbols indicating the position of the individuals highlighted in (C).

ever, analysis of individual animals showed that this was not the case. Instead, the majority of animals turned in random directions when stimulated with right wind [three examples in Fig. 4(A)] resulting in a broad distribution of turn accuracy of the population [Fig. 4(B)].

Escape Behavior Recovered by 6 Days after Cercal Crush. Six days after crush treatment, the escape behavior of the same 20 animals [Fig. 3(C)] recovered significantly ($p = 0.041$) from behavior demonstrated on PC1 [Fig. 3(B)]. However, treated animals did not recover the level of precision shown

by controls ($p < 0.001$). The mean percent correct responses for the population on PC6 (75.6 ± 2.95%) was significantly higher ($p = 0.001$, *t* test) than the mean percent correct responses on PC1 (39.7 ± 4.7%) [Fig. 3(D)]). However, the mean turn accuracy exhibited by the treated animals on PC6 remained significantly lower than that of individually marked controls (89.3 ± 3.9%; $p = 0.010$, *t* test).

Animals Recovered Their Escape Behavior to Varying Degrees. Some animals, such as the individual represented by circles in Figure 4(C), recovered to 100% turn accuracy, whereas a few individuals, such as the animal represented by triangles in Figure 4(C), did not recover at all. However, the turn behavior of the majority of animals showed a significant improvement by PC6 [Fig. 4(D)] but still did not attain the normal level of behavioral accuracy exhibited by control animals. The animal represented by the crosses exhibited this typical level of behavioral recovery [Fig. 4(C)].

Time Course of Behavioral Recovery. The time course of behavioral recovery was determined by testing a population of animals on each day following cercal crush (PC1–6). From day 7 on, animals started to molt; therefore, we could not test their behavior. The amount of data obtained on different days varied because of the random nature of sampling, the varying levels of the animals' activity on different days, and the need for removing animals from the test group because of appendage damage due to extensive testing and handling. Thus, we were unable to sample a sufficient number of data points on day 5. Figure 5 shows that whereas the animals' escape turns to wind from the treated side were almost randomly oriented during the first 3 days after treatment, turn accuracy suddenly improved to 70% by PC4 and did not improve further on PC6. This suggests that the reestablishment of synaptic connections largely took place between PC3 and PC4.

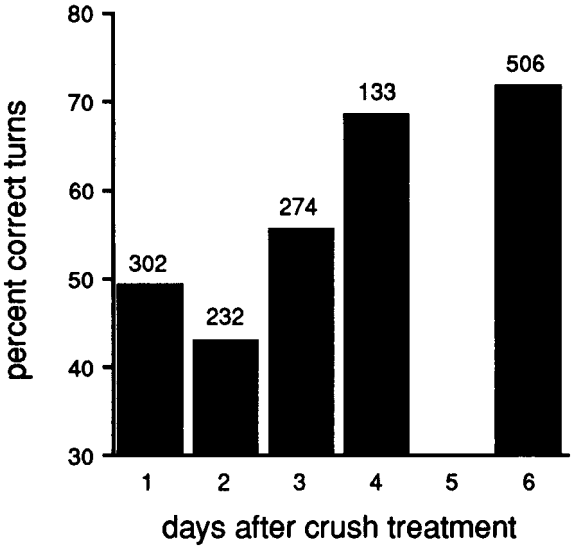


Figure 5 Time course of behavioral recovery. Turn accuracy of the total population of crush-treated cockroaches at different times after treatment. Data are expressed as a percentage of correct turns to wind stimuli from the right (treated) side. Owing to the random sampling method, group sizes varied daily (see text); *n* scores indicate group size. Escape behavior improved markedly between PC3 and PC4. Because we could not base this analysis on sufficient numbers of trials on individual animals, a statistical analysis was impossible.

Morphology of Regenerated Filiform Hair Afferents

To examine any morphologic correlates of behavioural recovery, afferents were stained intracellularly from PC1 to PC7. Sensory neurons filled immediately after the cercal nerve crush (PC0) appeared normal up to the crush site. Here the axon ended abruptly. This was found in all treated animals tested ($n = 4$), thus confirming the success of the crush procedure. By PC1, the axon had retracted almost to the point where it entered one of the two cercal nerve tracts [see Fig. 7(A)]. The distal end of the axon was thickened and had a clublike shape. On PC2, some neurons showed signs of outgrowth, such as filopodia and an elongated axon that had begun to grow along the appropriate cercal nerve tract [see Fig. 7(A)]. By PC3, most neurons exhibited this morphology, and some of them had already reached the terminal ganglion where they started to form an arborization [Figs. 6(B) and 7(B)]. In some preparations, both L and M of the right cercus were filled. In all these cases, both neurons had regenerated to a similar extent. On PC4, the majority of neurons had regenerated into the terminal abdominal ganglion and formed arborizations within the cercal glomerulus [Fig. 7(B)]. Some filopodia were still visible on PC5 and, more rarely, on PC6. From day 5 onward, the typical afferent blebs started to appear.

The pattern of growth of the 21 M and 22 L neurons examined on PC6 and 7 can be classified into four types.

Type 1. A total of 27.3% of the L neurons and 14.3% of the M neurons filled on PC6 or later belonged to this type. The arborization was almost

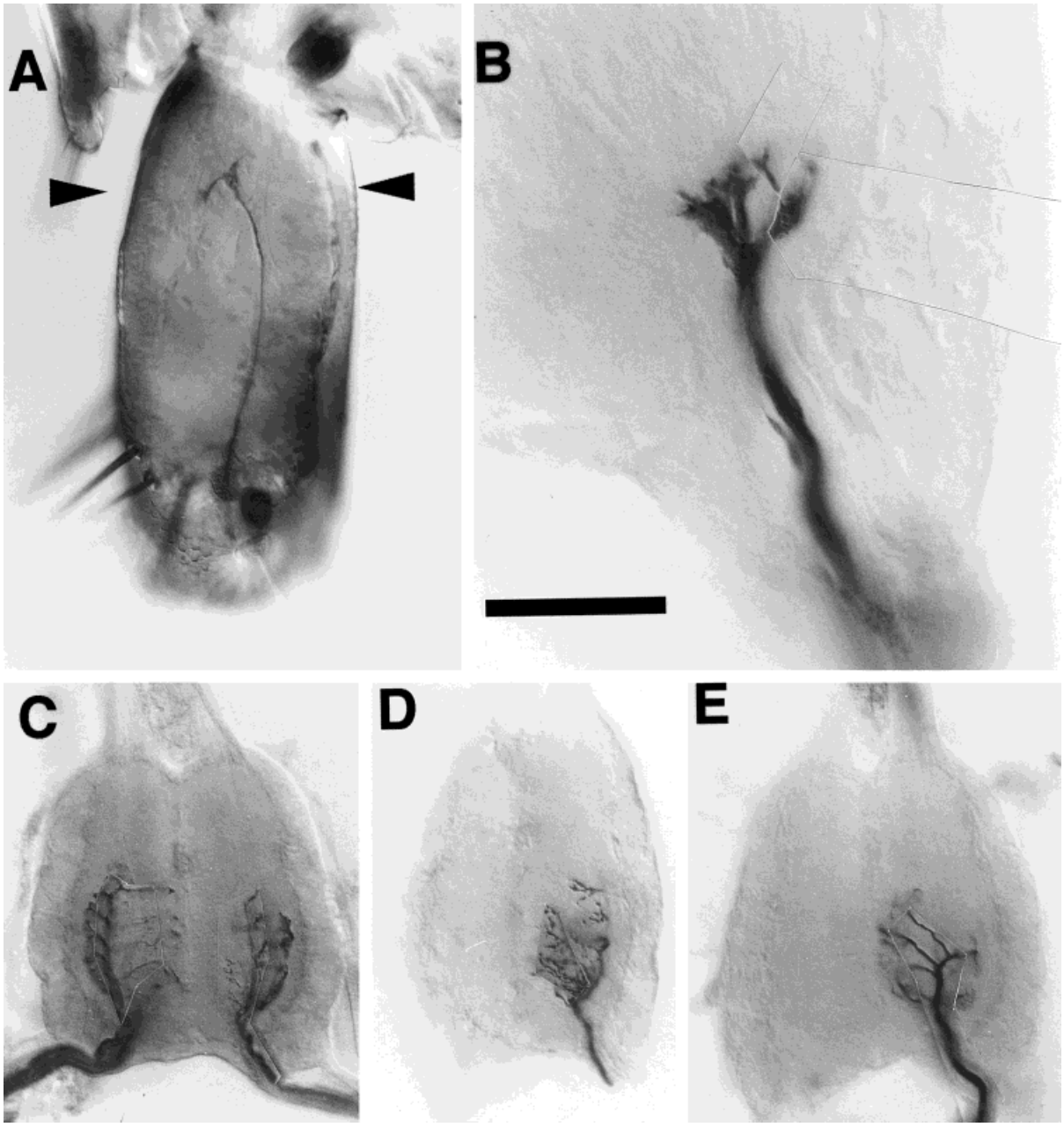


Figure 6 Most crushed afferents regenerated into the CNS; a few did not. (A) Ventral view of a right cercus 6 days after crush (crush site indicated by arrowheads). The stained medial neuron was extending into filopodia but did not manage to cross the crush site. (B) A right medial neuron 3 days postcrush entered the cercal glomerulus and began to form an arborization. (C) Six days postcrush, a regenerated right L neuron formed a mature arborization that bore some resemblance to the unlesioned L neuron on the left. (D) A medial neuron, 6 days postcrush, formed an arborization very different from the normal M-type branching pattern [compare with Fig. 9(A), left]. Note the numerous terminal swellings characteristic of mature SN arborizations. (E) M neuron 6 days postcrush, closely resembling the normal arborization. Scale bar: (A,C,D) = 100 μm; (B) = 20 μm.

normal. The main axon took the same course as in controls, and primary branches emerged at their characteristic position, as reported by Blagburn and Thompson (1990) and Blagburn et al. (1996). However, some primary branches could be missing [Figs. 6(E) and Fig. 8(A), second from left; Fig. 9(A),

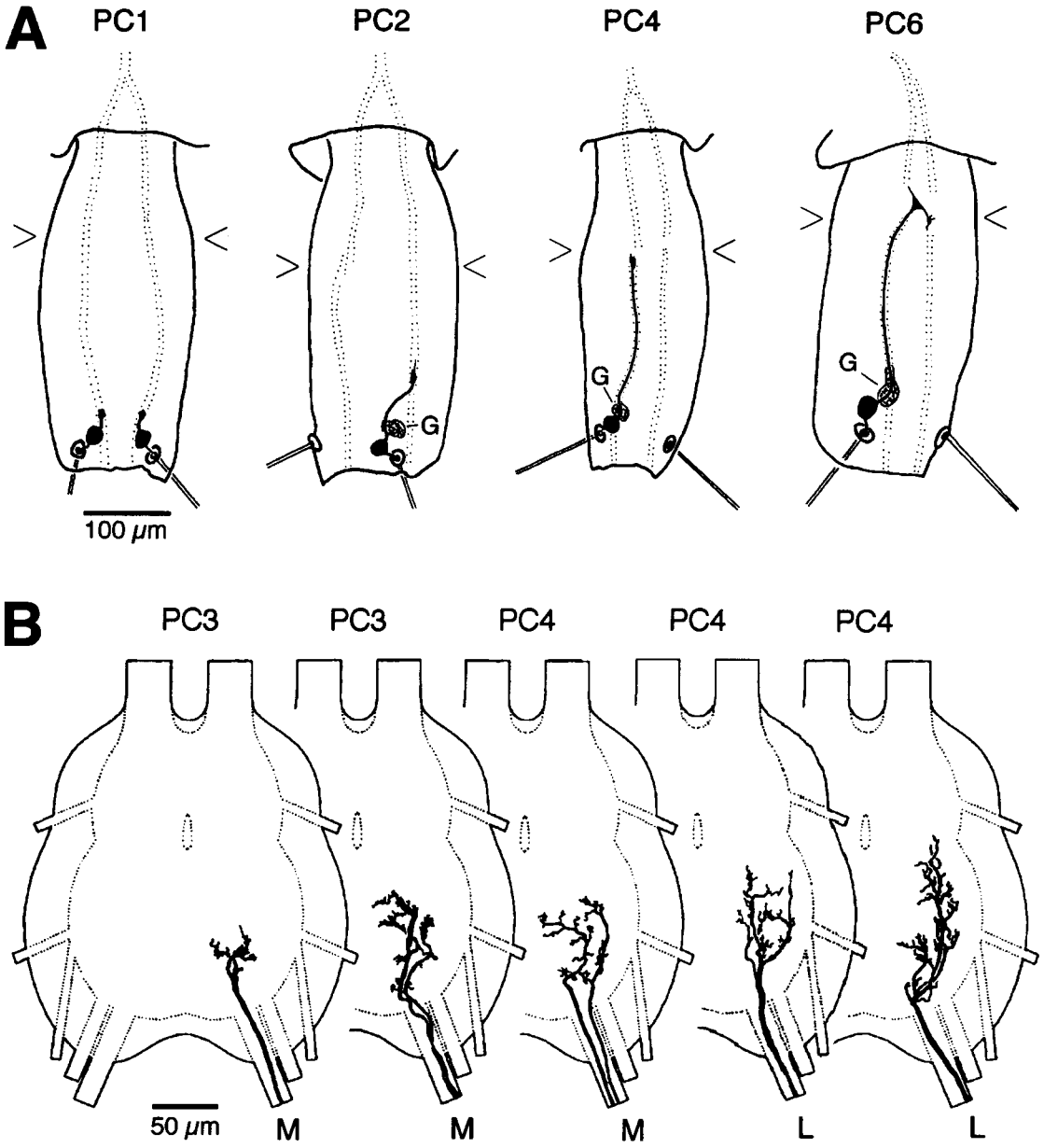
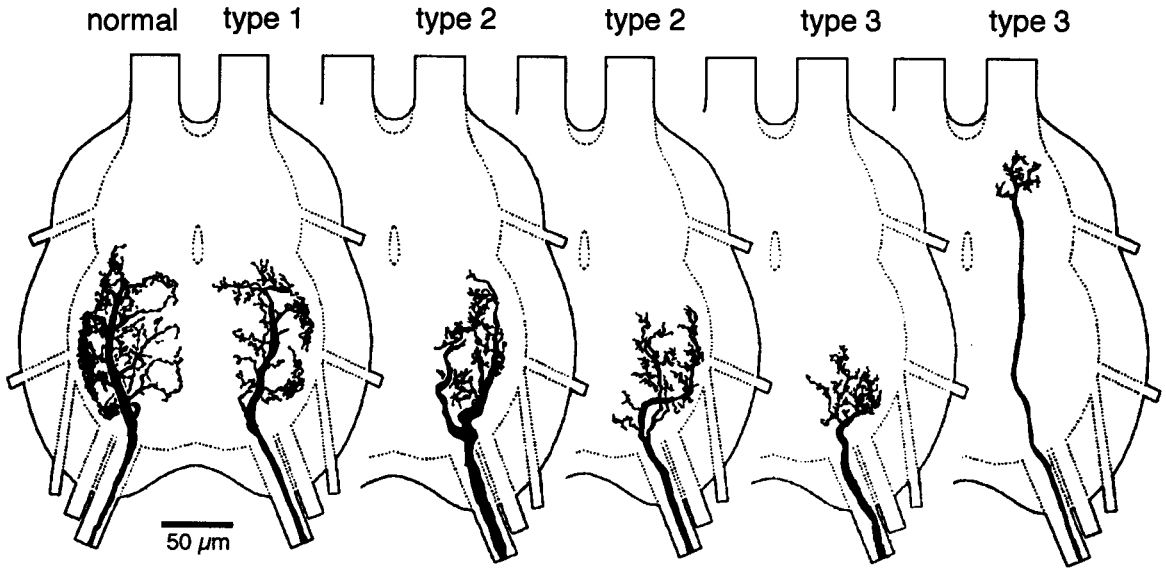


Figure 7 Most regenerating neurons entered the terminal ganglion 3 days after treatment. (A) Drawings of four right cerci (dorsal view) with one or two sensory neurons stained. On day 1 postcrush (PC1), both M and L retracted to the point of entry into the cercal nerve tracts (dotted). On PC2, an L neuron started to grow. An L neuron stained on PC4 and an M neuron stained on PC6 failed to grow across the crush site (indicated by arrowheads). G = glia cell. (B) Drawings of three M neurons and two L neurons filled with Lucifer yellow 3 or 4 days after treatment (indicated above). Neurons began to form an arborization in the terminal ganglion which bore some resemblance to their normal morphology. Note the bifurcated axons in the second and third preparation from the left.

second from left]. The most medial arborization of normal L neurons which runs longitudinally to delineate the medial extent of the cercal glomerulus [Fig. 8(A), left] never appeared in regenerated cells.

Type 2. A total of 36.4% of the L neurons and 42.9% of the M neurons belonged to this type. The axon took a course that was very different from normal, but the neuron still established an arboriza-

A



B

	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
GI1	0	P		0	0	P		P			0	
GI2	0	0	0	0	0	0	0	0	0	0	0	0
GI3	+	+	+	+	—	+	—	+	—	+	—	—
GI4	+	+		+	+	+	—		—	+		—
GI5	0	0		0	0	0					0	
GI6	+	+	+	+	+	+	+	+	+	+	—	—

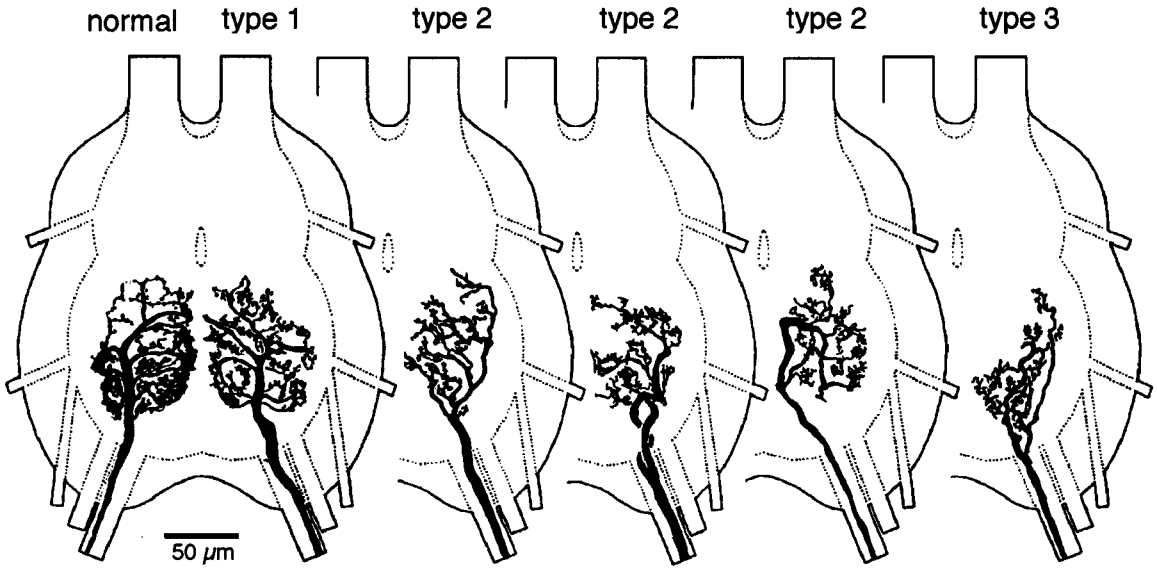
C

	control	PC1	PC6	PC1	PC6	PC1	PC6	PC1	PC6	PC1	PC6
% correct	100	n.d.	100	17	80	n.d.	n.d.	n.d.	n.d.	29	50
n	5	0	2	12	10	0	0	0	0	17	4

Figure 8 Morphology of regenerated L neurons correlated with input to GIs and escape behavior. (A) Drawings of five regenerated L neurons filled with Lucifer yellow 6 days after treatment, and a normal L neuron (left) from an untreated animal. For explanation of types, see the text. (B) Table of synaptic connections from corresponding neurons in (A) to GIs with a cell body ipsilateral (ipsi) and contralateral (contra) to the regenerated neuron. The experiment was performed by sequentially recording activity in as many GIs as possible, while mechanically stimulating the lateral filiform hair (+ = monosynaptic connection; P = polysynaptic connection; 0 = no connection, as expected; − = connection missing: blank = not determined). (C) Table of behavioral responses of the animals containing the corresponding neurons in (A) given as the percentage of correct turns away from wind from the right on PC1 and PC6. The number of trials is given below. n.d. = not determined. The behavioral responses of the animals with the neurons shown third from left and extreme right are illustrated in Figure 12.

tion within the cercal glomerulus [Fig. 6(C,D)]. The regenerating M neuron in particular only rarely followed the original trajectory with its characteristic medial bend at the level of the A9 segmental commissure. Arborizations, however, were formed mainly in the dorsal and medial portion of the cercal glomerulus by regenerated M neurons [Fig. 9(A)] and in the ventral and lateral portion by L neurons [Fig. 8(A)], as found in controls. In some preparations, the regenerating neuron had a medially directed branch originating from the point of entry into the neuropil [Fig. 8(A), third from left; Fig. 9(A), third from left], similar to those of most second-instar sensory neurons on cercal segments 4–6 (Thompson et al., 1992). The neurons apparently did not invade regions other than the cercal glomerulus and did not cross the midline. In several cases (19%), the M axon bifurcated within the cer-

A



B

	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
GI1	+	+					+	—	—	+	—	
GI2	+	+			—	+	+			+	—	
GI3	0	+				—		+	0	+	0	
GI4	+	+				+			—	+	—	+
GI5	0	+				+	0	+	0	—	0	
GI6	0	+			0	+		+	—	—	0	+

C

	control	PC1	PC6	PC1	PC6	PC1	PC6	PC1	PC6	PC1	PC6
% correct	90	n.d.	n.d.	n.d.	n.d.	50	91	38	87	25	78
n	10	0	0	0	0	4	11	8	15	8	18

Figure 9 Morphology of regenerated M neurons correlated with input to GIs and escape behavior. (A) Drawings of five regenerated M neurons filled with Lucifer yellow 6 days after crush, and a normal M neuron (left) from an untreated animal. (B) Table of synaptic connections of the corresponding neurons in (A) to GIs. Procedure and abbreviations as in Figure 9. (C) Table of behavioral responses of the animals containing the corresponding neurons in (A).

cal nerve before it reached the ganglion to produce its arborization from two parallel axons [Fig. 7(B), second and third from left; Fig. 9(A), third from right]. This was only rarely seen in regenerating L neurons.

Type 3. A total of 22.7% of the L neurons and 28.5% of the M neurons belonged to this type. The axon grew into the terminal ganglion, but arborizations were formed within <50% of the appropriate target area. They were limited to the most posterior part of the cercal glomerulus [Fig. 8(A), second from right], or (in one case) to the most anterior neuromere of the terminal ganglion with the axon bypassing the cercal glomerulus [Fig. 9(A), right]. In the former case, it was not clear whether the neuron was at an early stage of regenerating its arborization or had actually stopped growing; in the latter case, it would be very similar to the 6k afferent on segment 6 of the second instar cercus (Thompson et al., 1992).

Type 4. A total of 13.6% of the L neurons and 14.3% of the M neurons belonged to this group. The axon did not regenerate into the terminal ganglion. The axon stopped at the crush site in the cercal nerve or may have grown back toward the opposite cell body following the other axon tract

within the cercus [Figs. 6(A) and 7(A), right]. In other cases, the sensory neuron either had no axon or only a very short axonal stump.

Neurons belonging to type 1 and 2 often lacked the most anterior and medial parts of the arborization. For that reason, these neurons exhibited only minimal overlap, if any, with the limited ipsilateral dendritic fields of GI1, GI2, and GI3.

Electrophysiology

Sensory neuron cell bodies impaled immediately after cercal crush showed normal membrane potentials of −60 mV or lower and action potentials of up to 15 mV [Fig. 10(A)]. Regenerated axons exhibited action potentials of up 40 mV recorded in the terminal ganglion [Fig. 10(B)]. In many cases, the spontaneous activity in regenerating neurons was less than in controls, but in contrast to the situation in adult crickets (Chiba and Murphey, 1991), the neurons could normally be induced to spike by depolarizing pulses or by moving the sensory hair. This allowed us to monitor synaptic connections when they first formed.

On PC1–2, no GIs received filiform input from the treated side ($n = 6$). This further confirms the efficacy of our crush procedure. In 1 out of 12 preparations tested on PC3, the L-contralateral GI6 synapse had regenerated (the sensory neuron was not successfully stained in this preparation). In 50% of the preparations tested between days 4 and 5 ($n = 12$) and 81% tested on PC6 and later ($n = 42$), at least some of the GIs received input from the crushed side [Fig. 10(B–D)]. Not all of the monosynaptic connections were reestablished with equal probability (Table 1). GIs with their contralateral arborization on the treated side were much more reliably reconnected than the GIs with their ipsilateral arborization. Among the ipsilateral GIs, GI1–3 were reconnected only rarely, whereas GI4–6, which have more extensive and more caudally located ipsilateral dendrites, were reconnected more often (Table 1). There was good correlation between lack of synaptic input to ipsilateral GIs 1–3 and A a lack of SN arborization in the anteriormedial part of the cercal glomerulus. Ipsilateral GI2 was more often reconnected than GIs 1 and 3. This correlates with the fact that GI2's neurite, which bears its ipsilateral dendrites, is more posterior than the other two in the terminal ganglion. GI2's primary neurite crosses the midline in the A9 commissure, about 50 μm posterior to the neurites of GIs 1 and 3, which run in the A8 commissure (Blagburn, 1989). In preparations where ipsilateral GIs 1–3 received cercal input, the regenerated sensory neuron had at least a few branches in the anteromedial part of the cercal glomerulus [L: Fig. 8(A,B), second from left; M: Fig. 9(A,B), third from right].

Incorrect monosynaptic connections were not observed. This is difficult to determine for GI1, which does not receive monosynaptic input from L, but does receive indirect polysynaptic input from contralateral L (Hill and Blagburn, 1996) as well as from L-type afferents in the second instar (Thompson et al., 1992). In two regenerated preparations, GI1 received L input from the treated side [Figs. 8(B) and 10(D)], which was judged to be polysynaptic because of the long latency of the e.p.s.p. (10.23 ± 0.825 ms, $n = 22$) to the stimulus. Monosynaptic contralateral M-GI1 e.p.s.p. is 4.12 ± 0.145 ms ($n = 30$) under the same stimulus conditions. The latency of monosynaptic e.p.s.p.s in regenerated preparations was not different from controls.

In the majority of preparations, the e.p.s.p.s at the regenerated synapses had approximately the same amplitudes as those reported for untreated animals (Table 2), indicating that the appropriate synaptic strengths were reestablished. An exception is the M-contralateral GI2 synapse, where the average e.p.s.p. reaches just 2.18 ± 0.31 mV, which is about 40% of the normal size. Occasionally, very large monosynaptic e.p.s.p.s (>10 mV) [Fig. 10(C)] were recorded between L and GI3 and M and GI2.

Electron Microscopy

Some crustacean and leech axons are capable of surviving long periods separated from their cell bodies, and regeneration involves fusion of proximal and distal axon segments (Hoy et al., 1967; Carbonetto and Muller, 1972; Bittner, 1991). We therefore carried out electron microscopy of the terminal ganglion after nerve crush, to confirm that the distal portions of the filiform afferents did indeed degenerate. Electron microscopy of the filiform hair afferents within the terminal ganglion showed that 3 h after crushing the nerve, the axons were significantly more electron dense than normal axons [Fig. 11(A,B)]. By 12 h PC, the axons were shrunken and electron dense, and often surrounded by glial processes and abutted by other, unidentified, cell profiles containing evidence of phagocytic activity [Fig. 11(C)]. These morphologic changes are characteristic of type 1 degeneration of insect nerve terminals (Schürmann, 1980; Brandstätter et al., 1991). By PC2 the axons had disappeared completely from the terminal ganglion neuropil, leaving

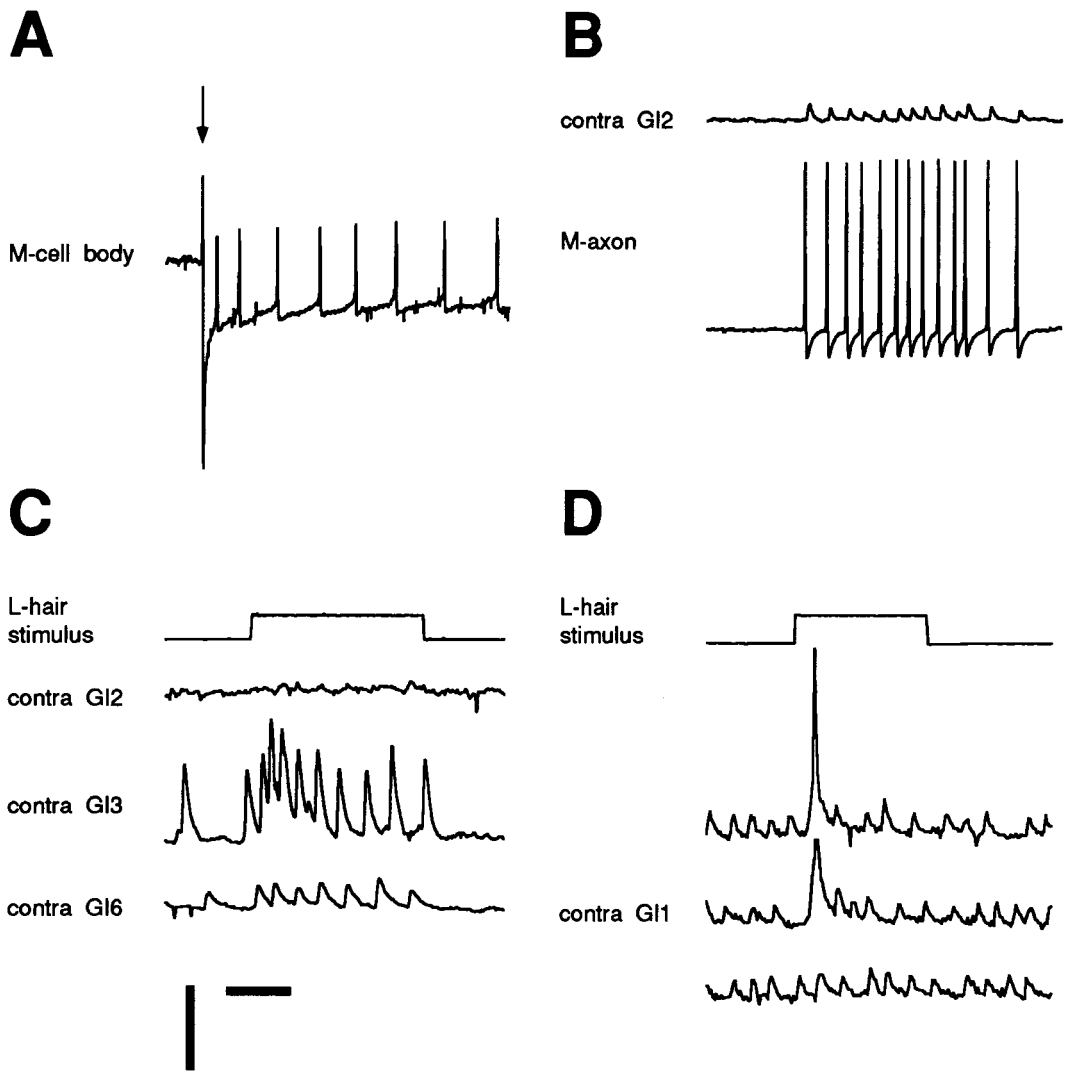


Figure 10 The majority of regenerating sensory neurons reestablished specific synaptic connections. (A) Action potentials recorded from the soma of an M neuron 1 day postcrush, elicited by release from −1 nA hyperpolarization (arrow points to switching artifact). (B) Paired intracellular recording from the axon of a regenerated M neuron on PC6 in the terminal ganglion and the contralateral GI2 soma. Spikes in M evoked e.p.s.p.s in GI2. (C) Examples of e.p.s.p.s recorded from GIs in a regenerated preparation on PC6. Pushing the lateral sensory hair (upper trace) elicited no response in contralateral GI2, abnormally large e.p.s.p.s in contralateral GI3, and normal e.p.s.p.s in contralateral GI6. Traces are from recordings from the same preparation under the same stimulus conditions. (D) In another preparation on PC6, polysynaptic input to GI1 is demonstrated. Moving the L hair evoked either no response (bottom trace) or an e.p.s.p. with an onset latency of approximately 10 ms (middle trace), which sometimes gave rise to an action potential (upper trace). The morphology of the regenerated L neuron is shown in Figure 8, third from the left. Horizontal scale = 40 ms; vertical scale = 10 mV, except for (B) (lower trace = 20 mV).

behind no cellular debris or gaps in the neuropil which could have been used as guidance cues by the regenerating axons [Fig. 11(D)].

In addition, electron microscopy was used to confirm that the synaptic connections formed by regenerated afferents with the GIs were indeed monosynaptic, and that the patterns of connections were specific. To this end, GIs were injected with HRP. In

Table 1 Regenerating SNs Connect More Likely to Contralateral Than to Ipsilateral GIs

	GI1	GI2	GI3	GI4	GI5	GI6
Ipsilateral	[25%]	23.1%	10.5%	41.7%	[38.5%]	55.9%
	(8)	(14)	(19)	(24)	(13)	(34)
Contralateral	73.3%	70%	52.8%	88.9%	64.7%	68.4%
	(15)	(20)	(36)	(18)	(17)	(38)

The percentage of GIs receiving synaptic input from regenerated SNs on PC6. Contralateral GIs 3, 4, and 6, and ipsilateral GI4, which received input from both L and M, were regarded as being reconnected when at least one of the connections (usually the stronger one) was detected. Ipsilateral GIs 1 and 5 received only polysynaptic input (percentage in square brackets). The numbers of preparations from which each GI was recorded are given in parentheses.

many cases, it was not possible to identify the regenerated afferents because of their small size and aberrant positions. In favorable preparations, however, the axons occupied approximately their original positions in the neuropil and were sufficiently large to be identified. In these preparations, the L and M axons had type 1 morphologies which could be distinguished by the characteristic locations of their branches. Figure 11(E) illustrates one such preparation, in which GI1 was injected with HRP. GI1 dendrites selectively contacted the M axon and not the L, and formed synapses with it which had a normal morphology [Fig. 11 (insert)].

Analysis of Individuals Shows That Synapse Reformation Is Necessary for Behavioral Recovery

In all behaviorally tested animals in which e.p.s.p.s in at least some GIs were observed, the escape behavior had improved on PC6 (see examples in Figs. 8 and 9). The behavior of a representative animal, in which neuronal regeneration was observed by recording e.p.s.p.s from GIs and dye-filling a sensory neuron [Fig. 8(A), third from left], is shown in Figure 12 (triangles). While it exhibited random turn responses on PC1 [Fig. 12(A)], it turned more consistently away from right wind on PC6 [Fig. 12(B)]. In cases where the sensory neurons had not regenerated or arborized in the wrong area of neuropil [Fig. 8(A), right], there was no filiform hair input to the GIs [Fig. 8(B)] and the escape behavior remained disturbed on PC6 [Fig. 8(C)]. The behavior of this animal is represented as circles in Figure 12. The behavioral response to wind from the right had not improved by PC6; the animal turned into the wind in half the trials.

DISCUSSION

Escape Behavior Is Mediated by a Simple Set of Neurons

The first-instar cockroach is difficult to catch. This is because the four filiform hairs on its cerci, which detect even the slightest air movement, are specifically tuned to particular wind directions. The accuracy of the escape behavior relies on the specificity of the connectivity in the neuronal circuit. In the cricket cercal system, specificity of connections between afferents and interneurons may be achieved, at least in part, by anatomic separation of afferents with different directional preferences (Bacon and Murphey, 1984). In the cockroach, however, synaptic specificity relies on local cell–cell recognition events, since the arborizations of the sensory

Table 2 Most Regenerated SN–GI Connections Achieve Normal Synaptic Strengths

	e.p.s.p. [mV]	S.E.M.	*n*	Control
L-contra GI3	4.02	0.57	19	5.6
L-contra GI6	2.35	0.31	14	2.8
L-ipsi GI6	2.00	0.23	16	2.1
M-contra GI1	3.01	0.59	7	4.3
M-contra GI2	2.18	0.31	13	5.0
M-contra GI3	1.40	0.29	5	1.4

Average monosynaptic e.p.s.p. sizes at regenerated SN–GI synapses on PC6. Control e.p.s.p. sizes from untreated animals are taken from the literature (Blagburn, 1989).

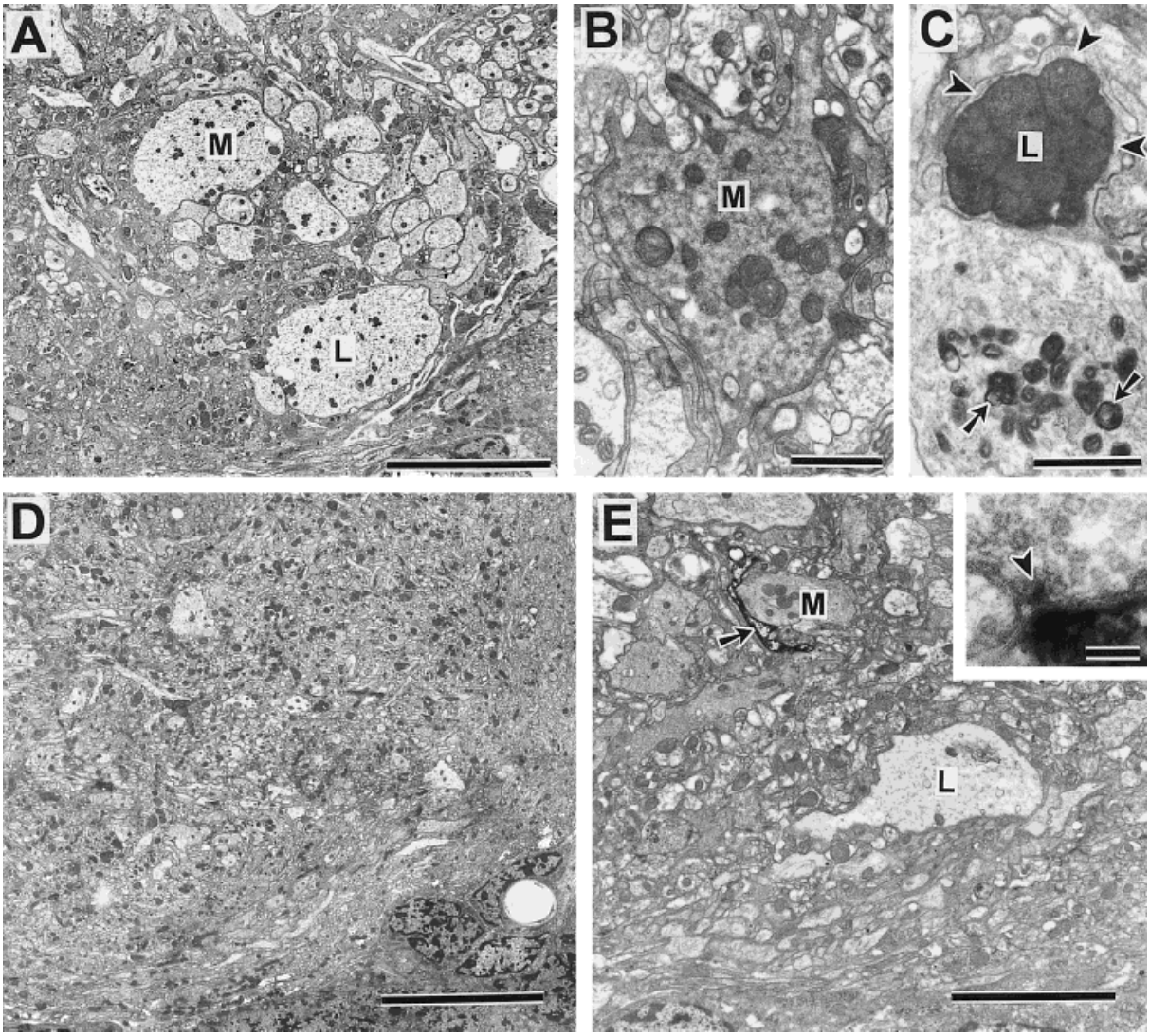


Figure 11 Electron microscopy showed that the filiform afferents degenerated completely and reconnected specifically. (A) Transverse section of the ventrolateral portion of the terminal ganglion neuropil showing lateral (L) and medial (M) filiform hair sensory axons. Anterior is up and lateral to right. Scale = 10 μm. (B) Branch of M afferent (M) 3 h after the cercal nerve was crushed. The cytoplasm showed increased electron density. Scale = 1 μm. (C) Branch of L afferent 12 h after nerve crush (L). The cytoplasm was greatly reduced and highly electron dense. The degenerating profile was surrounded by a glial process (arrowheads) and was adjacent to a cell profile containing secondary lysosomes (arrows). Scale 1 = μm. (D) An area of neuropil similar to that shown in (A), 2 days after nerve crush. The cercal afferent axons were completely absent and there were no large extracellular spaces or cellular debris remaining. Scale = 10 μm. (E) Regenerated lateral (L) and medial (M) afferents 6 days after nerve crush. The M afferent was contacted by a dendrite of GI1 which had been injected with horseradish peroxidase (arrow). Scale 5 = μm. (Inset) High-power view of a typical dyadic synapse formed by M with the GI1 dendrite and another, unidentified process, showing the characteristic presynaptic bar (arrowhead). Scale = 100 nm.

neurons are not anatomically separated (Blagburn and Thompson, 1990). For example, the M sensory neuron makes monosynaptic cholinergic connections with the GI2 interneuron, but the L neuron fails to make a connection with GI2 despite the fact that GI2 makes transient filopodial contacts with the L axon during embryogenesis (Blagburn et al., 1996).

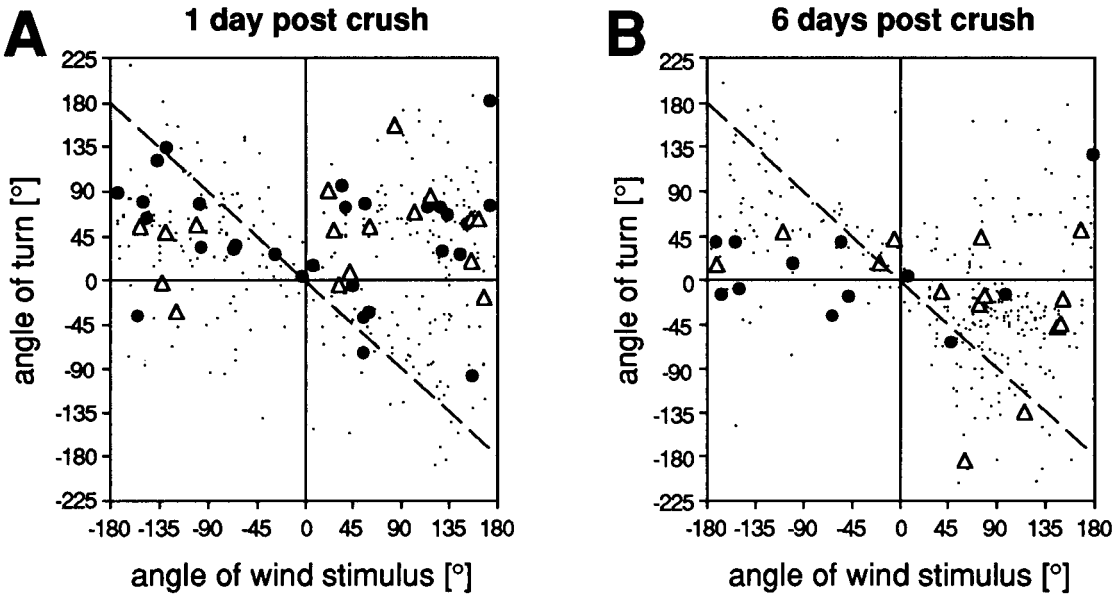


Figure 12 Behavioral case studies of individual animals. Data are the same as those presented in Figure 3(B,C) with the escape responses of two representative animals (circles and triangles) highlighted against the cloud of other animals' responses on both days. (A) On PC1, individual animals exhibited both correct and incorrect turn orientations. (B) By PC6, individuals either demonstrated more correct turns than on PC1 (triangles) or executed the same number of correct and incorrect turns (circles). Morphology and synaptic connectivity of the L neuron in these individuals is shown in Figure 8, third from the left (triangles) and right (circles).

Escape Behavior Is Perturbed after Crushing the Base of a Cercus

The present study shows clearly that 1 day after the base of a cercus had been crushed, first-instar cockroaches exhibited perturbed escape behavior. Instead of turning away from an air disturbance, they turned randomly, and often toward the stimulus. Similar results were obtained by Comer and Dowd (1987) by either removing one cercus from adults or transsecting one connective of the ventral nerve cord in late nymphs.

In our experiments on first instars, crushing the base of the right cercus axotomized right M and L afferents. Because these afferents respond to wind coming predominantly from the right (Dagan and Volman, 1982), any excitation of the remaining left afferents will inevitably create the perception of left wind, leading to an escape turn to the (treated) right-hand side. The experiments of Dagan and Volman (1982), in which both hairs were plucked from one cercus, conformed perfectly to this expectation: Winds from the untreated side were responded to appropriately, whereas winds from the lesioned side led to a consistent turn into the wind. A laminar wind stimulus from the right rear quadrant should not, however, elicit any escape response, because it ought to move the left M and L hairs in their inhibitory direction (Dagan and Volman, 1982). The turning responses in the study of Dagan and Volman may have been due to their low-intensity (0.3 m/s) horizontal wind stimuli, which might have created turbulences leading to (inappropriate) excitation of the hair afferents on the left cercus in response to wind from the right (lesioned) side. In our experiments, in which the base of the right cercus was crushed, a higher velocity (0.7–0.8 m/s) wind was applied from 45°; this stimulus may have been more laminar than that of Dagan and Volman and may therefore have reduced excitation of the left cercal afferents to wind from the treated side. Instead, the slightly stronger winds used in our study might have elicited escape responses mediated by the antennae (Stierle et al., 1994). The antennal input is normally insignificant because of the lower-threshold, shorter-latency response mediated by the cercal hairs. For adult cockroaches, the threshold for the antennal response lies between 1.2 and 1.8 m/s (Stierle et al., 1994). The wind intensities used in our study might have been just above the threshold for the antennae-mediated response in the much smaller first instar, but provided poor directional information which resulted in randomly oriented escape turns.

It is clear that the randomly oriented responses observed in our study were not due to an incomplete crush procedure. If crushing had been inconsistent, the population would have consisted of a group of unsuccessfully treated animals turning correctly and a group of successfully treated animals turning in the wrong direction. However, analysis based on individual animals shows that this was not the case; the majority of individuals produced both correct and incorrect responses. Further, electrophysiology and filling of afferents on PC1 confirmed the reliability of the crush procedure in general, and all dye fills of afferents in behaviorally tested animals revealed novel arborizations indicative of regeneration. Despite the fact that our data differ from those of Dagan and Volman (1982), the crush treatment used in this study does perturb escape behavior significantly.

Behavior Recovers to Near Normal after Regeneration

Six days after crush treatment, the accuracy of the perturbed escape behavior improved again to near normal. The behavioral accuracy was very similar on days 4 and 6. We were unable to test beyond day 6, because the animals began to molt from PC7 onward. The largest improvement was observed between PC3 and PC4. This correlates well with our anatomic and electrophysiologic findings; regenerating neurons start to arborize in the cercal glomerulus and form synapses with GIs at this time. This correlation is most likely causal, because animals in which the afferents failed to reconnect to the GIs showed no sign of behavioral recovery. Other phenomena, such as the partial recovery of behavior after complete cercal removal (Vardi and Camhi, 1982), take much longer. Thus, regeneration of the filiform afferents is necessary for recovery of escape behavior. However, although afferent regeneration is sufficient to produce correctly oriented escape runs most of the time, the behavior of regenerates does remain distinguishable from that of untreated animals.

Why Does the Recovered Behavior Remain Distinguishable from Normal?

Six days after treatment, the recovered escape behavior was still less accurate than normal. This can be explained by a number of our electrophysiologic findings: (a) Although no inappropriate connections were observed, on no occasion did we see the full complement of normal connections reestablished. In particular, ipsilateral synaptic connections to the ventral GIs (1–3) were less likely to re-form than contralateral connections. These GIs are particularly important for escape behavior (Ritzmann, 1981; Comer, 1985). (b) Although the strengths of the synaptic connections that had been reestablished appeared to be relatively normal, a consistent feature was the small size of the M to contralateral GI2 synapse. These changes in connectivity presumably led to a perturbation of the normal GI receptive fields, resulting in behavior that did not fully recover. We did not gather enough data to correlate the number of reestablished synapses with the percentage of correct turns. Nevertheless, it seems that the reestablishment of only a subset of the normal synaptic connections was sufficient to mediate reasonably accurate escape behavior.

Why Is the Morphology of the Regenerated Neurons Different from Normal?

Within 4–7 days, the crushed filiform hair afferents regenerated into the appropriate locations within their target neuropil, the cercal glomerulus. Within the cercus, the axon followed one of the two cercal nerve tracts, as normal. Within the terminal ganglion, however, the main axons often followed trajectories different from the original. Aberrant morphology seems to be a common feature of regenerating insect sensory neurons, since it is also reported from the cricket cercal system (Chiba and Murphey, 1991) and the locust auditory system (Lakes and Kalmring, 1991). The distal segments completely disappeared by 2 days, thus excluding the possibility that regenerating axons reconnect to surviving distal segments as can happen in the leech S cell or crayfish giant axons (Bittner, 1991). Also, there appeared to be no holes left behind in the neuropil or glial channels which could have been used as guidance cues by the regenerating axons. However, we cannot exclude that glia cells or extracellular markers still remained.

The aberrant morphology of the regenerated afferents could be explained by either environmental changes in the terminal ganglion, which obscure the normal pathways, or loss of the neurons' ability to respond to some of the environmental cues. The postembryonic environment into which the afferents regenerated differed considerably from the embryonic environment they encountered when they origi-

nally developed at about 50% of embryogenesis. The embryonic terminal ganglion neuropil is longer and narrower than in the first instar. When the afferents establish their arbors in the embryo, the five neuromeres of the terminal ganglion are just beginning to fuse (Blagburn et al., 1996). The longitudinal pathways in the neuropil are thus much closer together, and this, coupled with the larger amounts of extracellular space, would make their guidance cues easier for the afferents to encounter. In the first instar, the neuropil is both wider and more tightly packed, factors which would reduce the likelihood of regenerating filiform afferents finding their previous cues, assuming that these still exist. That possibly causes some of the regenerated sensory neurons to bear some resemblance to second-instar neurons, which normally grow into the terminal ganglion within the time period in which the regeneration experiments were carried out. Severing the cercal afferents causes degeneration in the cercal glomerulus. Thus, after cercal crush, the normal postembryonic environment in the terminal ganglion differs considerably from normal. This could be tested by examining the morphology of late second-instar neurons growing *de novo* into the deafferented cercal glomerulus. If the cercal glomerular environment changed as a result of degeneration, this ought to be reflected in the altered morphology of these second-instar afferents.

However, the morphologic variability of the regenerated afferents was not reflected in the pattern of connectivity. Although the full complement of the normal SN–GI connections was never observed after regeneration, the connections that did regenerate were always appropriate. This was also the case on days 3 and 4 after cercal crush, when the synapses started to form. We could not detect any intermediate inappropriate connections that later retracted. However, it cannot be excluded that tiny incorrect connections were transiently formed, as has been shown for the embryo (Blagburn et al., 1996), that were indetectable with our methods. Even if there would had been such transient inappropriate connections, however, they would not have had an effect on the behavior. We restricted our analysis to the GIs. It is possible that regeneration of connections to other interneurons (e.g., local wind-sensitive neurons) is less accurate. However, we did observe that the normal indirect polysynaptic inputs to ipsilateral GI1 and 5 were reestablished.

Therefore, synaptic specificity persists during regeneration in the first instar. This result is very similar to that seen by Chiba and Murphey (1991), who studied the regeneration of cricket cercal afferents. It also confirms the finding of Blagburn et al. (1991), that SN morphology is not a determinant of connectivity in this system.

Why Do the Connections Regenerate with Different Probabilities?

Not all connections were reestablished with the same probability; in perticluar, the ipsilateral ventral GIs (1–3) were reconnected only rarely. This can be explained by the lack of overlap between incompletely regenerated afferents and the small ipsilateral dendrites of GI1–3, which are restricted to the more anterior regions of the neuropil. It appears that the less extensive and the more anterior the dendrites of particular GI are, the less probable is a successful regeneration of the synapse. Obviously, a lack of overlap prevents synaptic connection. It is more difficult to explain why in many cases SNs fail to reestablish the full pattern of anatomically possible connections despite extensive overlap, at least as judged at the light-microscopic level.

In the embryonic cercal glomerulus, postsynaptic GI dendrites grow toward the presynaptic M and L axons (Blagburn et al., 1996). Recently, it was directly shown that dendritic filopodia actively initiate synaptogenesis rather than being a passive target (Ziv and Smith, 1996). We have no evidence that the first-instar GI dendrites are capable of filopodial exploration in response to deafferentation. In contrast to the situation in the embryo, it is the presynaptic afferents which are forced to wander through a more densely packed neuropil in search of their postsynaptic partners. This may result in a lower probability of such encounters.

Future Prospects

Our ultimate aim in investigating this preparation is to determine the underlying mechanisms that control synaptic specificity between the SNs and their GI targets. In the present study, we have shown that during regeneration in the first instar, the same level of synaptic specificity is achieved as during unperturbed embryogenesis. We have recently suggested that the cockroach *engrailed* gene may play a role in determining the specificity of these synapses (Blagburn et al., 1995). The regeneration paradigm established in the present report could be used as a postembryonic testbed for the perturbation of *engrailed* expression to examine this hypothesis.

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