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Ectopic sensory neurons in mutant cockroaches compete with normal cells for central targets

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Summary

The cercus of the first instar cockroach, *Periplaneta americana*, bears two filiform hairs, lateral (L) and medial (M), each of which is innervated by a single sensory neuron. These project into the terminal ganglion of the CNS where they make synaptic connections with a number of ascending interneurons. We have discovered mutant animals that have more hairs on the cercus; the most typical phenotype, called "Space Invader" (SI), has an extra filiform hair in a proximo-lateral position on one of the cerci. The afferent neuron of this supernumerary hair (SIN) "invades the space" occupied by L in the CNS and makes similar synaptic connections to giant interneurons (GIs). SIN and L compete for these synaptic targets: the size of the L EPSP in a target interneuron GI3 is significantly reduced in the presence of SIN. Morphometric analysis of the L afferent in the presence or absence of SIN shows no anatomical concomitant of competition. Ablation of L afferent allows SIN to increase the size of its synaptic input to GI3. Less frequently in the mutant population, we find animals with a supernumerary medial (SuM) sensillum. Its afferent projects to the same neuropilar region as the M afferent, makes the same set of synaptic connections to GIs, and competes with M for these synaptic targets. The study of these competitive interactions between identified afferents and identified target interneurons reveals some of the dynamic processes that go on in normal development to shape the nervous system.

Key words: insect, CNS, development, synaptic competition, cockroach, sensory neuron.

Introduction

Insect sensory systems provide favourable material for tackling problems in developmental neurobiology. Insect sensilla are formed in the periphery by individual epidermal cells going through rounds of mitosis to form clones of cells, which collaborate to make the elements of the sensillum. For example, in a typical mechanoreceptor, the tormogen cell secretes the cuticle of the socket, trichogen secretes the cuticle of the hair shaft, the third cell differentiates into a neuron and the fourth cell differentiates into the accessory cell (Bate, 1978). The sensory neuron axon navigates into the CNS where it forms specific synaptic connections with first order interneurons (Bacon and Murphey, 1984) and motoneurons (Weeks and Jacobs, 1987).

One way to study the development of these systems is to perturb them: cuticular structures and the attendant sensory neurons can be transplanted to ectopic locations, ablated or even created de novo as the result of surgery. Murphey and his co-workers have successfully applied these techniques to the cricket, *Acheta domesticus*, and a dynamic picture has emerged in which sensory neurons are somehow able to "read" their positional values in the periphery and thereby form arborizations in appropriate regions of the CNS (Murphey, 1985; Kämper and Murphey, 1987). Once there, afferents targeted to the same regions of the CNS compete for postsynaptic partners (Murphey, 1986; Shepherd and Murphey, 1986).

As an alternative to scissors and scalpel, mutants can be sought where the normal pattern of sensory projection is altered. In our routine studies of the cockroach, *Periplaneta americana*, we discovered first instar animals that bore one or two extra filiform hairs on their cerci. Each supernumerary hair is innervated by a single sensory neuron. Since the wild-type animal only has two filiform hairs on its cercus (Blagburn and Beadle, 1982), the phenotypic change observed in these mutants represents a dramatic increase in the filiform hair afferent input to the terminal ganglion, without surgical manipulation. In this study, we investigate the effect of the larger number of afferents on the strength of the synaptic connections to the postsynaptic interneurons. We find that the supernumerary afferents compete with the normal afferents for synaptic space on identified interneurons. The system continues to be dynamic postembryonically because ablation of single afferents increases the synaptic strength of the remaining afferent connections. This decrease and

increase in synaptic strength takes place in the absence of any anatomical change of the afferent. A preliminary account of these findings has already appeared (Bacon and Blagburn, 1989).

Materials and Methods

First instar cockroaches, *Periplaneta americana*, obtained from our wild-type and mutant colonies, were used in all experiments.

Scanning electron microscopy

The posterior abdomen, with cerci attached, was removed from cold-anaesthetised animals and fixed overnight in 5% glutaraldehyde in phosphate-buffered saline (pH 7.2) at 4°C, washed in distilled water, postfixed in 1% osmium tetroxide solution for 2 hours and dehydrated through graded ethanols. After critical-point drying and sputter coating, specimens were viewed in a Jeol JEM 100C SEM.

Staining of afferents and interneurons

The heads and legs were removed from cold-anaesthetised animals, which were immobilised ventral surface uppermost in insect wax. A drop of insect saline confined by petroleum jelly was placed on the animal to prevent desiccation. A thin-walled microelectrode was filled with 2.5% cobalt chloride, then the tip was broken by gently touching it with the tip of another microelectrode (this latter step proved critical to produce optimal staining). The microelectrode, held steady in Plasticine, was inserted into the cuticular cupola at the base of the target hair. A shallow angle of insertion maximized the chance of impaling the more proximally located filiform hair neuron cell body. Preparations were left for 4 hours at room temperature in a humid atmosphere. After removing the distal two cercal segments, dorsal cuticle and gut, the preparation was treated with ammonium sulphide, washed thoroughly in saline, and fixed in Carnoy's for 1 hour.

To stain an afferent and one of its target interneurons simultaneously, the method employed by Blagburn (1989) was used. Briefly, neurons were stained by iontophoresis from microelectrodes filled with 3% hexammine cobalt chloride by passing 3-5 nA depolarizing current pulses of 500 ms duration, at a frequency of 1 Hz. GI cell bodies were injected for approximately 6 minutes. Hair neuron cell bodies were exposed by dissecting open the proximal segment of the cercus, and were injected for approximately 20-30 minutes, followed by a diffusion period of similar duration. Ammonium sulphide was added to the saline (2 drops in approximately 2 ml of saline), followed by several washes in saline. The tissue was fixed for 1 hour in 4% formaldehyde in 0.1 M phosphate buffer with 3% sucrose, then transferred to 4% formalin in methanol for 30 minutes.

The terminal ganglion and cerci of preparations made by either method were dissected and silver-intensified (Bacon and Altman, 1977). After mounting in Canada Balsam, the stained arborization could be unequivocally traced back to its identified hair. Selected whole-mounts were photographed and drawn, then the ganglia were removed from the slide, and embedded in a "soft" Araldite-Medcast (Ted Pella Inc.) mixture. Ganglia were sectioned at 10 μm, using fragments of razor blade stuck to glass knives with cyanoacrylate glue and mounted in a Sorvall ultramicrotome. Serial sections were mounted in Canada Balsam and drawn, allowing reconstructions of the neurons to be made. Because of the variable shrinkage undergone by the ganglia during dehydration, drawings were adjusted to scale using standard outlines of live ganglia viewed with Nomarski optics, or of resin sections of ganglia fixed in glutaraldehyde and osmium tetroxide.

To quantify any effects on the L arborization by the presence of SIN, we measured the total surface area of the main L axon and its medial branches, and of its anteriormost medial branch alone. Camera-lucida drawings were made of silver-intensified cobalt fills of L afferents from mutant animals, both in the presence and absence of SIN. The drawings were digitized and the number of filled pixels measured using "Image" software on a Mac IIci computer. This count was converted to units of area (in μm^2) and multiplied by π to give the surface area of the equivalent cylinders. L afferents were not identified as being on control or SIN sides until after the analysis was completed.

Electrophysiological recording

Methods for isolating the CNS and cerci for electrophysiological recording have been described previously (Blagburn, 1989). To confirm the origin of the SIN EPSPs, the SI hair was left mobile and its tip was stuck to a broken micropipette coated with petroleum jelly; this was mounted on a loudspeaker which was driven by a pulse generator. Movement of the hair selectively stimulates the underlying sensory neuron, producing a burst of spikes in the afferent axon and consequently a burst of EPSPs which can be recorded in any postsynaptic cells (Fig. 4B).

To allow the unequivocal identification of the small M EPSPs in the contralateral GI3, ipsilateral GI2 was impaled with a microelectrode. Large M EPSPs could be recorded in the ipsilateral GI2, and these served as markers for the small M EPSPs in contralateral GI3, allowing the amplitudes of the latter to be measured. In the presence of SIN, it was not possible to measure these small M EPSPs amongst the other larger EPSPs and so the L and SIN synapses were silenced by ablating their cell bodies with finely sharpened forceps after first removing the two distal cercal segments (Fig. 4C).

Ablation of the L sensory neuron

The cell body of L was ablated soon after hatching by breaking off the two distal segments of the SI cercus and inserting finely sharpened forceps into the lumen. This procedure did not affect the cell body of M, and caused no damage to the more proximal SIN soma. The tip of the cercus was allowed to seal with clotted haemolymph, and the animals were left for 7 to 10 days before electrophysiology was carried out. Ablation of the L cell body causes L EPSPs to disappear immediately, and the L axon shows signs of degeneration after 24 hours (J.M. Blagburn and R.E. Blanco, unpublished observation).

Results

The occurrence of supernumerary cercal hairs is a mutant phenotype

There are two filiform hairs on the ventral side of the normal first instar cercus, one lateral (L) and one medial (M). Our inbred laboratory colony produces some animals with supernumerary hairs. The most common variant that we observe are animals with one cercus bearing an extra Space Invader (SI) hair, on the proximal cercal segment, usually situated half way between the base and the row of bristles, in the same circumferential position as the normal L hair (Fig. 1). These animals represent approximately 2% (65 out of 3181 screened animals) of our laboratory inbred colony. By selecting and crossing such animals to produce an F_1 population,

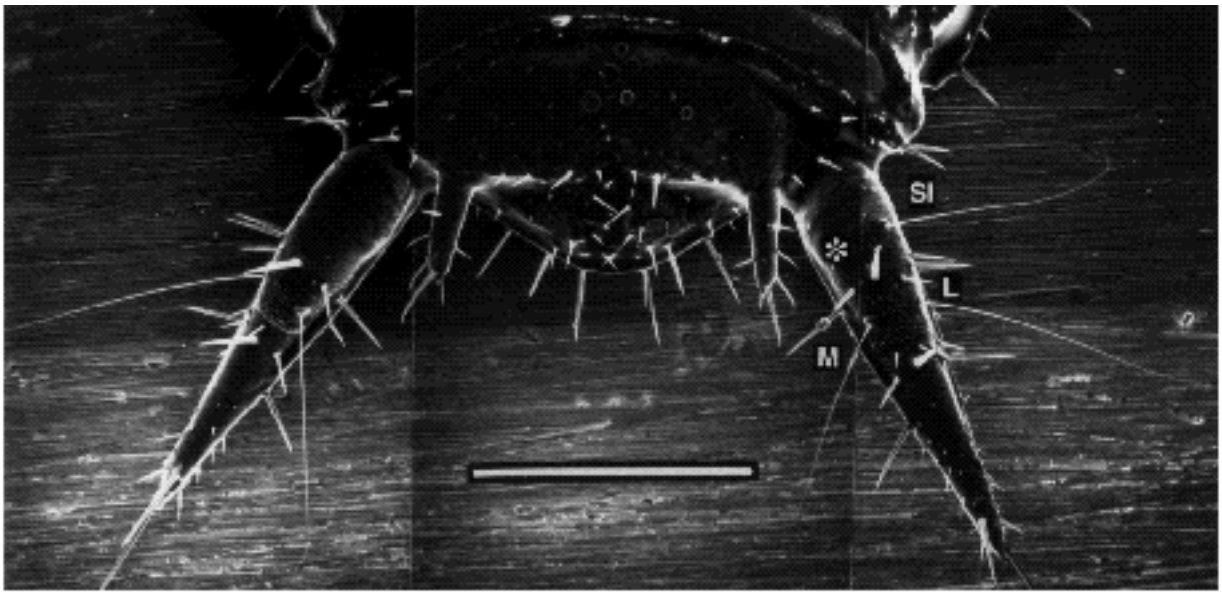

Fig. 1. Mutant Space Invader cockroaches have supernumerary hairs on their cerci. In this first instar individual, S.E.M. of the ventral posterior region shows its two cerci. The animal's right cercus has the normal complement of two filiform hairs but its left cercus has a "Space Invader" (SI) hair as well as the normal lateral (L) and medial (M) hairs. Neither cercus bears the supernumerary medial hair but its usual position is shown by *. Scale: 500 μm.

we have increased the occurrence of this variant to approximately 10% (47 out of a sample of 451). Further selection has produced an F_2 comprising 18% SI individuals (155 out of 838). At this stage, two colonies were established by selection of animals with or without extra hairs. Selection of SI individuals produced an F_3 comprising 25% SI (578 out of 2311) whereas the animals without an extra hair produced an F_3 of 17% SI (425 out of 2565). The increase and decrease of SI occurrence in response to selection reveals a genetic basis to this phenomenon and it is therefore appropriate to call SI a mutant phenotype. Whether it is polygenic or results from incomplete penetrance of a single-gene mutation has yet to be determined.

Selective inbreeding of this colony has also caused the appearance of animals with other supernumerary hairs on their cerci. Less commonly than the SI hair, we find animals with a supernumerary medial hair (SuM) at a similar proximal position to the SI but at approximately the same circumferential position as M (Fig. 1). Very rarely, hairs are found on the cercal midline (Blagburn et al., 1991).

The afferents of the supernumerary hairs project to the same neuropilar regions as the corresponding normal hairs

The morphology of the normal L and M afferents have been described previously (Blagburn and Thompson, 1990). Briefly, both the L and M axons project anteriorly within the terminal ganglion neuropil, and form medially and dorsally directed branches which grow around the margins of the cercal glomerulus (Figs 2, 3). A major difference between the two is that M forms a very characteristic 45° medial bend half way along its length while L continues to the anterior end of the glomerulus. The main L axon ranges from 5 - 10 μm in diameter, while that of M has a diameter of 4 - 8 μm. L gives rise to 5 or 6 large dorsally directed branches which form dense clusters of varicosities in the dorsolateral region of the glomerulus, while M has only two dorsally directed branches. M gives rise to 4 or 5 large medial branches which, along with the main axon, curve around the medial border of the glomerulus and, together with the dorsal branches, arborize profusely around the dorsal margin of the posterior half of the cercal glomerulus. L, in contrast, has 4 - 6 thinner medially directed branches which curve around the ventral margin of the cercal glomerulus. Therefore, the second major anatomical difference between the two afferents is their appearance in transverse section: M forms a characteristic circum-glomerular arborization, and L occupies mainly the ventro-lateral margins of the glomerulus. Neither axon forms extensive branches within the core of the glomerulus, apart from some small branches lying in a horizontal plane in the centre. L extends a small, anteriorly projecting branch out of the cercal glomerulus, sometimes reaching as far as the A7 neuromere, while M forms a small anterior branch which remains within the confines of the glomerulus.

The SI hair is innervated by a single sensory neuron (SIN) and SuM innervates the supernumerary medial hair. SIN closely resembles the L afferent and arborizes in the same region of neuropile (Fig. 3). The arborization of SIN is somewhat sparser than that of L, with the main axon being approximately 3 - 6 μm in diameter. Although SIN has the same number of side branches as L, these appear to form fewer varicosities. The anterior extra-glomerular branch projects further into the A7 neuromere than that of L, while the medial branches do not extend as far around the glomerulus. The arborization of SuM is similar to that of M and arborizes in the same neuropilar region, although it has a slightly smaller axon diameter (3 - 6 μm) and forms few, if any, medial branches (Fig. 3). It rarely gives rise to the small anterior branch. Instead, one or more anteriorly projecting dorsal branches sprout from the axon at the point where it enters the

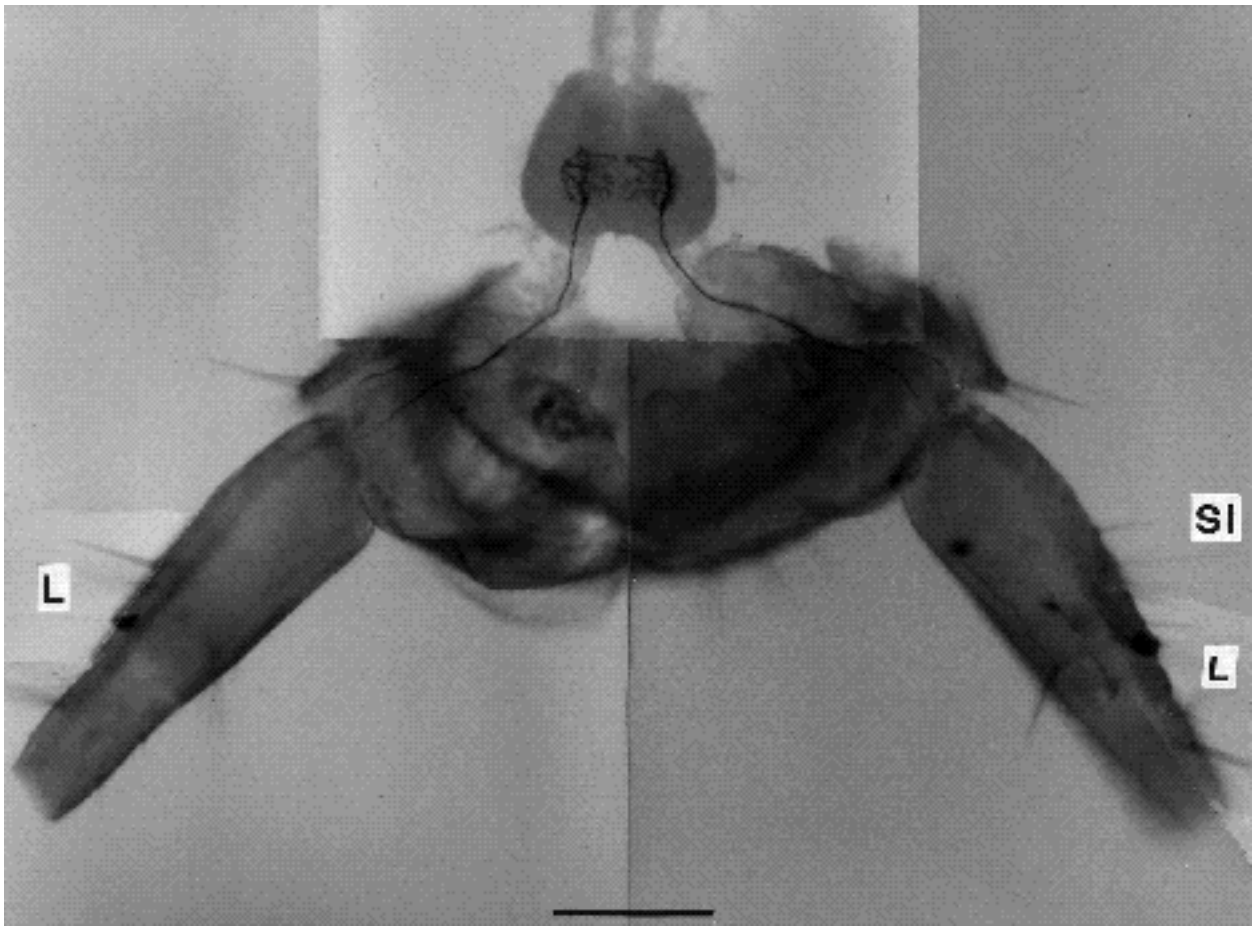

Fig. 2. Cobalt filling reveals the entire structure of filiform afferents from the cell body, beneath the hair on the cercus, to the afferent arborization within the terminal ganglion. Ventral view of the two cerci, posterior cuticle and terminal abdominal ganglion. The L afferent on both the normal cercus (left) and the cercus with an SI hair (right) has been stained. The distal ends of the cerci have been removed to enhance intensification of the afferent cell bodies. It is difficult to intensify stained axons more proximally in the cercus. Scale: 200 μm.

cercal glomerulus and project to the anterior end of the glomerulus.

The anatomical similarities between the supernumerary afferents and some of the second instar afferents (Thompson et al., 1992) suggest that the mutant phenotype may result from the precocious differentiation of one or two second instar sensilla. However, examination of individual SI cerci before and after ecdysis shows that the second instar also has the extra sensillum, which demonstrates that the hair is truly supernumerary.

Do the supernumerary afferents compete with the normal afferents for synaptic targets?

Electrophysiological recording has shown that SIN forms monosynaptic connections with the same subset of giant interneurons (GIs) as L in the terminal ganglion: ipsi- and contralateral GI3 and GI6; SuM synapses with the same GIs as does M: ipsilateral GIs 1 and 2 and contralateral GIs 1, 2, 3, 5 and 6 (Blagburn et al., 1991). Since SIN mimics L and SuM mimics M in terms of arborization and connectivity within the CNS, we are afforded a unique opportunity to investigate any interactions between mutant supernumerary afferents and normal afferents as they establish their central synaptic connections. We focused our attention on the connections from the lateral afferent pair to ipsilateral and contralateral GI3, and from the medial pair to ipsilateral and contralateral GI2. These monosynaptic connections have larger PSP sizes than the afferent connections to other interneurons and therefore provide the best opportunity to examine any changes in synaptic strength, which may occur as a result of synaptic competition, with the greatest precision.

In normal first instar animals, GI3 receives strong monosynaptic input from the ipsilateral and contralateral L and a very small input from the contralateral M (Figs 4A, 5A,B; Blagburn, 1989). Intracellular recording from GI3 in mutant animals reveals synaptic potentials evoked by both L and SIN (Fig. 4B). Immobilizing the L hair and then moving the SI hair to cause bursts of spikes in SIN shows that the potentials from SIN are smaller than those from L (Figs 4B, 5A,B), which is consistent with the sparser SIN arborization. In these mutant preparations, it is usually not possible to see the tiny M EPSPs in amongst the other, larger EPSPs. To reveal these, L and SIN were first silenced by ablating their cell bodies and ipsilateral GI2 was impaled. The large M EPSPs in ipsilateral GI2 act as markers for detecting the small EPSPs from the same afferent in contralateral GI3 (Fig. 4C).

Fig. 5A clearly shows that the average synaptic potential

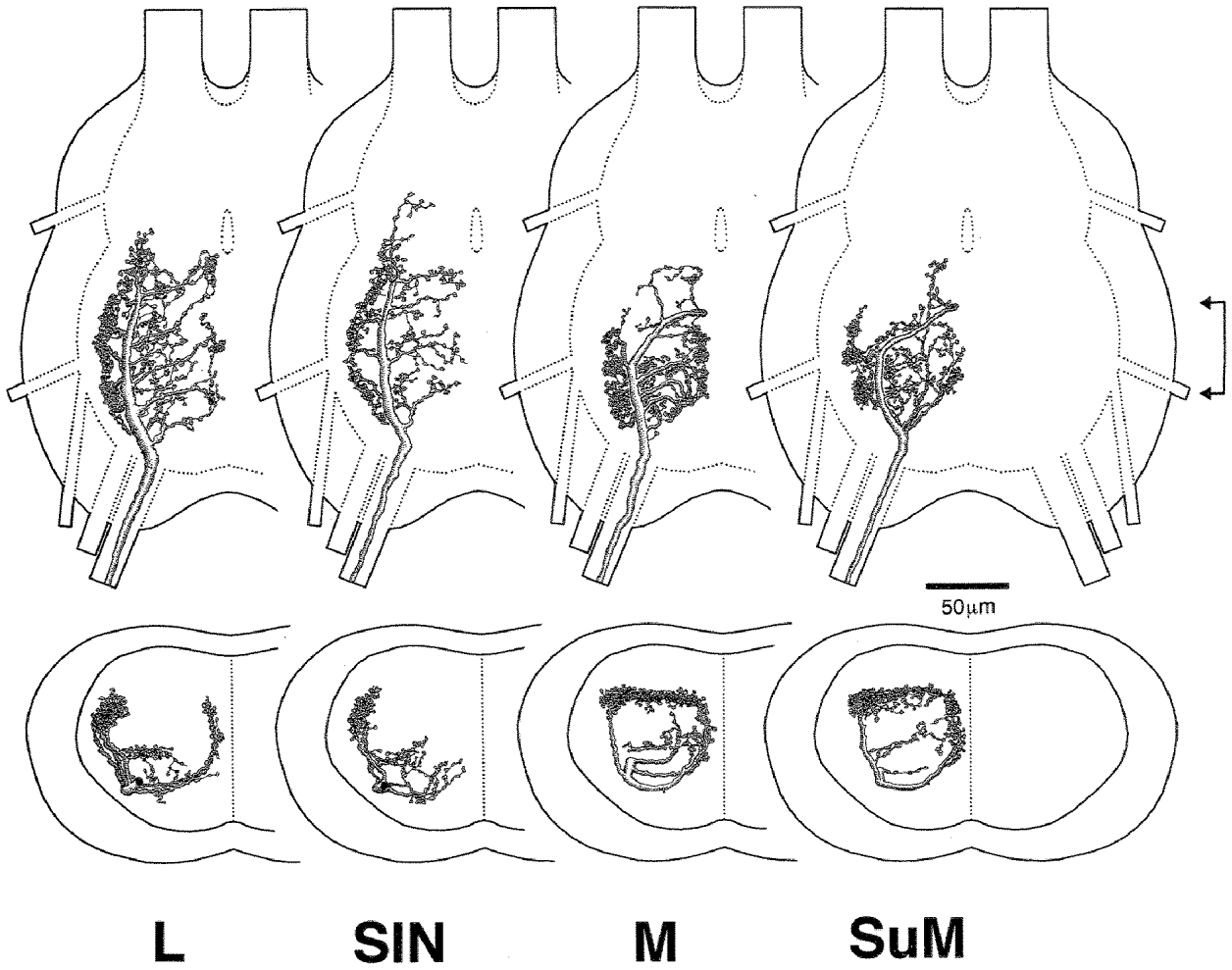

Fig. 3. Supernumerary afferents make similar central arborizations to their normal counterparts at the same cercal circumferential position. L, SIN, M and SuM afferents in the cockroach first instar terminal ganglion in ventral wholemount view (top) and transverse sections viewed from anterior (bottom). The transverse sections were reconstructed from the region delineated by the arrows. The arborizations of L and SIN project predominantly to the ventro-lateral region of the cercal glomerulus while M and SuM form their characteristic circumglomerular arborization with a concentration of dorsal varicosities.

amplitude from L onto ipsilateral GI3 is reduced from 4.45 ± 0.27 mV in wild-type animals to 3.28 ± 0.30 mV in the presence of SIN. Comparison of these values with a *t*-test showed that the difference is significant at the 1% probability level ($P < 1\%$). Similarly, L produces an average EPSP of 6.10 ± 0.25 mV onto contralateral GI3 in wild-type individuals (Fig. 5B) but this is reduced to 4.33 ± 0.36 mV when SIN is also present ($P < 1\%$). The weak synaptic connection made by M onto contralateral GI3 is affected even more by the presence of SIN (Fig. 5B); it is reduced from 1.43 ± 0.16 mV in normal animals to 0.68 ± 0.08 mV in SI animals ($P < 0.1\%$). Synaptic transmission in SI animals appears to be normal because L and M on the other cercus (without the supernumerary hair) form synaptic connections with GI3 of magnitudes which are not significantly different from wild-type: L forms 4.07 ± 0.18 mV EPSPs to ipsilateral GI3 and 5.89 ± 0.31 mV to contralteral GI3. M forms 1.34 ± 0.08 mV EPSPs to contralateral GI3. In addition, GI3s receiving supernumerary afferent input have similar input resistances (approximately 20 MΩ) to GI3s in wild-type animals. We conclude, therefore that the synaptic potentials from L and M on the cercus with the supernumerary hair are reduced in magnitude because of the presence of SIN.

The strength of these synaptic connections can also be modified postembryonically by surgery. L, the more accessible of the two lateral sensory neurons, was ablated on the SI cercus shortly after the animals' emergence, and the amplitude of the synaptic connections made by SIN to ipsilateral GI3 was found to have increased when measured 7-10 days later (2.88 ± 0.18 mV compared to 1.71 ± 0.13 mV in unoperated animals, $P < 0.1\%$; Fig. 5A), presumably as a result of reduced competition. The amplitude of the inputs to contralateral GI3 from the remaining SIN and M were also tested (Fig. 5B). These were found to be significantly larger (4.43 ± 0.41 mV and 0.97 ± 0.03 mV, respectively) than in the unoperated SI animals (2.12 ± 0.19 mV and 0.68 ± 0.08 mV, $P < 0.1\%$ and $P < 2\%$, respectively).

The effects of the presence of the SuM afferent on the strength of the synaptic connections between M and GI2 was also determined. To investigate this, intracellular recordings

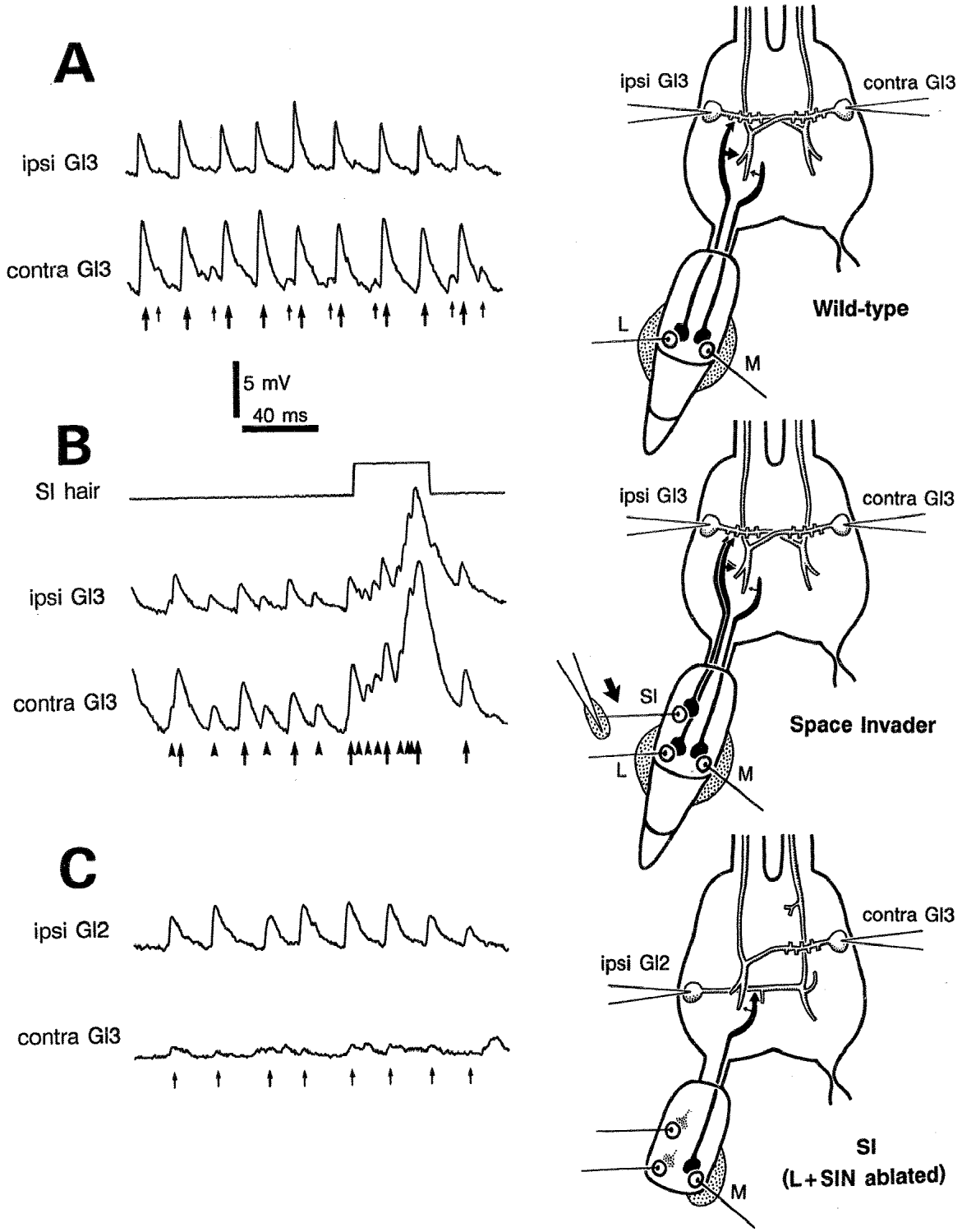

Fig. 4. Intracellular recording in the terminal ganglion shows that supernumerary afferents make synaptic connections. The right-hand cercal nerve was crushed in all of these experiments and the L and M filiform hairs were immobilized with petroleum jelly (stippled) which causes regular, tonic spiking in the afferents. The diagram to the right of each trace depicts the experimental situation. (A) Simultaneous recording in the ipsi- and contralateral GI3s in a wild-type animal. Large EPSPs (large arrows) occur synchronously in both GI3s; these result from the input of the regularly-spiking L afferent. Small EPSPs in the contralateral GI3 only (small arrows) arise from the M afferent. (B) In SI individuals, simultaneous recording in ipsi- and contralateral GI3s reveals a set of additional synchronous EPSPs (arrowheads) arising from SIN. Their identity is confirmed by moving the SI hair (displacement of top trace), inducing a burst of summating SIN EPSPs in both GIs, during which the larger L EPSPs (arrows) are visible. It is not possible to detect the small M EPSPs in contralateral GI3 amongst the larger EPSPs. (C) To allow the measurement of the small M EPSPs in the preparation shown in B, the L and SIN somata were ablated and the ipsilateral GI2 was impaled. The large EPSPs in GI2 can be used as markers for the occurrence of M EPSPs in contralateral GI3 (small arrows).

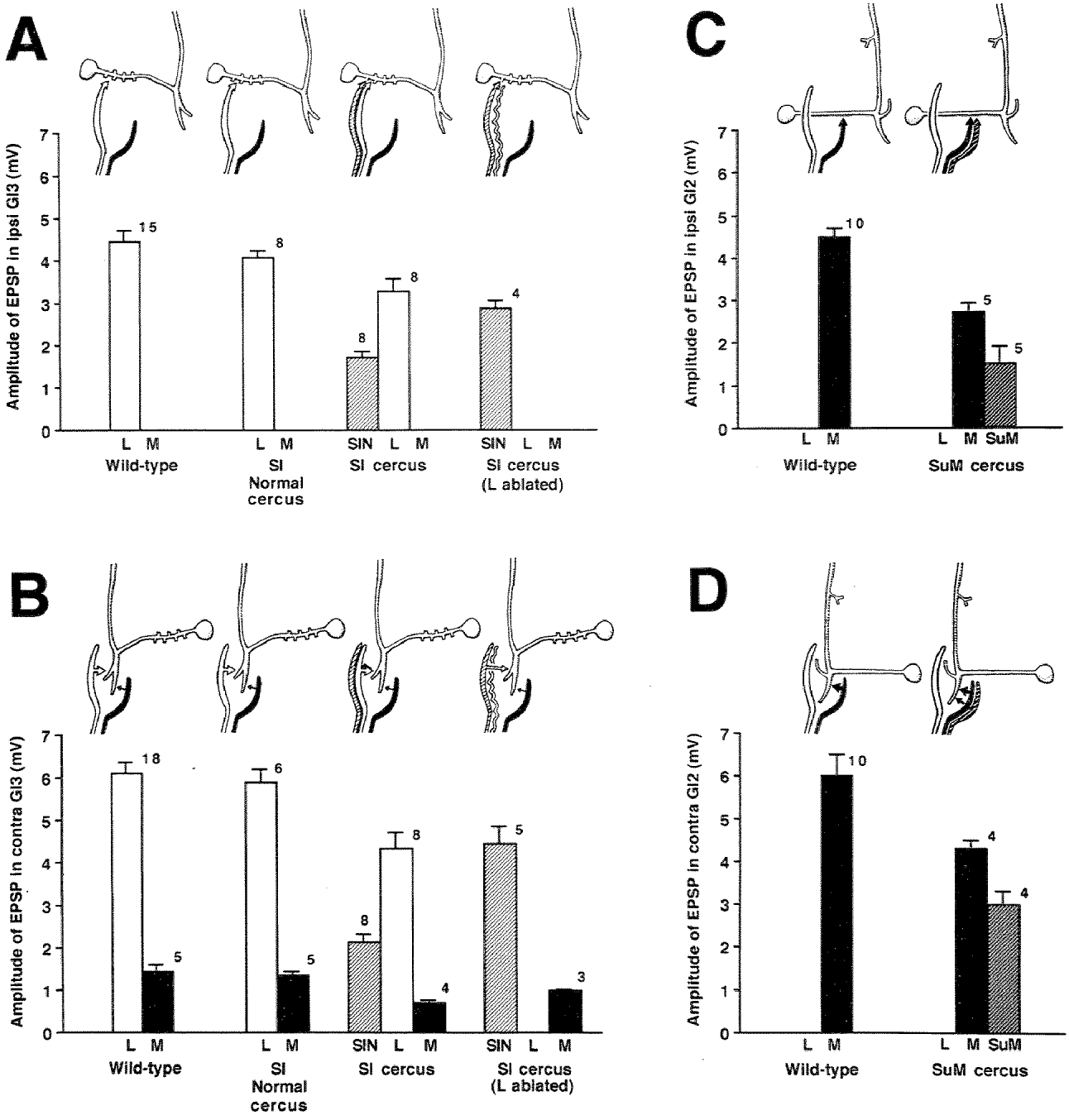

Fig. 5. Supernumerary afferents compete with normal cells for synaptic targets. Bars indicate the average EPSP amplitude from the afferent indicated on the horizontal axis. The numbers of preparations (from each of which at least 30 EPSPs were measured) are indicated. A. Averaged EPSP amplitude (in mV) in ipsilateral GI3 from the L and SIN afferent. There is no M - ipsi GI3 connection. There is no significant difference between the EPSP amplitudes from L onto ipsilateral GI3 in wild-type animals and those of the L afferent on the normal cercus of SI animals, whereas the amplitudes of the L EPSPs are significantly different in the presence of SIN. After ablation of L, the amplitudes of SIN EPSPs are significantly larger than in the unoperated SI animal. The diagram above each section of the bar chart depicts the neuronal configuration. B. The results in contralateral GI3 are similar to ipsilateral GI3 but there is input from M. The strength of its connection to contra GI3 is significantly reduced in the presence of SIN and increased significantly after L ablation. C. The presence of the SuM afferent significantly reduces the strength of the synaptic connections between the M and ipsilateral GI2. D. A similar result is seen in contralateral GI2.

were made from ipsilateral and contralateral GI2, and the amplitudes of EPSPs evoked by M in wild-type animals, M in the presence of SuM, and by SuM itself were measured. We did not have enough animals with a SuM hair to be able to carry out recordings of M EPSPs on the unaffected cercus. M EPSPs in the ipsilateral GI2 were reduced by the presence of SuM (4.5 ± 0.2 mV in wild-type compared to 2.7 ± 0.2 mV, $P < 1\%$; Fig. 5C). In contralateral GI2, the results also

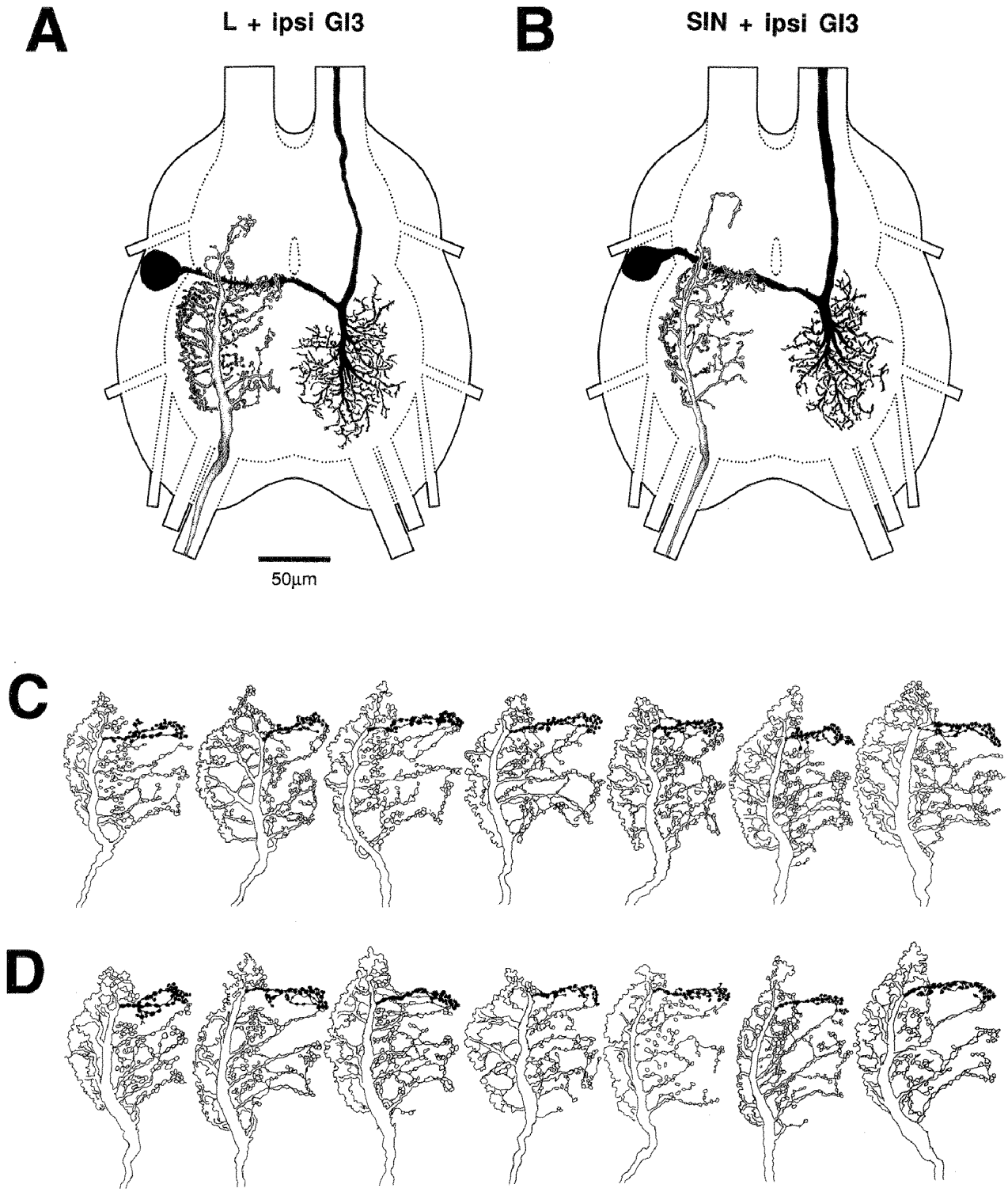

Fig. 6. There is no obvious anatomical concomitant of the synaptic competition. A. Drawing of simultaneous cobalt staining of L and ipsilateral GI3. Monosynaptic connections form between the anteriormost medial branch of the afferent and the ipsilateral branches and main neurite of GI3 (Blagburn, 1989). This identifiable branch was one of two regions of the arborisation chosen for quantification. B. The equivalent branch of SIN forms synaptic connections with ipsilateral GI3. C. L afferents in the presence of SIN. These were stained in seven SI animals on the cercus which did bear the supernumerary hair. The branch that makes monosynaptic connections to ipsilateral GI3 is shown in black. D. The same branch in seven L afferents in the absence of SIN is indicated. Quantification of these branches is given in Table 1, which shows that the presence of SIN makes no significant alteration to the arborization of L.

show that M EPSPs were reduced from 6.0 ± 0.5 mV in the wild-type animal to 4.3 ± 0.2 mV in the presence of the supernumerary afferent ($P < 5\%$; Fig. 5D).

Again, commensurate with the observation that SuM has a sparcer anatomy than M, SuM forms weaker connections with GI2 than M does: SuM produces 1.5 ± 0.4 mV EPSPs in ipsilateral GI2 and 3.0 ± 0.3 mV EPSPs in contralateral GI2 (Fig. 5C, D).

Is there an anatomical correlate of the competitive physiological interactions?

Does the presence of SIN affect the anatomy of L? The L afferent shown in Fig. 3 is from a wild-type animal so it gives no information about possible morphological changes of L in the presence of SIN. In contrast the preparation shown in Fig. 2 has stained examples of both a normal L and an L in the presence of SIN. Quantitative comparisons of such complex three-dimensional arborizations are difficult; in the cricket, a convenient measure of the extent of afferent arborizations has been the counting of axonal varicosities (Murphey, 1986). However in the first instar cockroach a significant part of the filiform afferent's arborization consists of a dilated axon and thick side branches, which cannot be readily divided into varicosities but which nevertheless make large numbers of output synapses to GIs and other neurons (Blagburn et al., 1985). Apart from counting the number of synaptic contacts directly, total process surface area probably represents the most meaningful measure of arborization size.

First, we focused our attention on the anteriormost medial branch of L that probably mediates most of the monosynaptic connections to ipsilateral GI3 (Fig. 6A; Blagburn, 1989). SIN has an equivalent branch which presumably also mediates most of its connections to ipsilateral GI3 (Fig. 6B). The fact that this branch is easily identified, and that synaptic competition has been shown to exist at this connection (Fig. 5A) make this an obvious branch for quantification. Second, we analysed the entire ventral half of the L arborization, that is, the main axon and the medial branches which run horizontally across the ventral surface of the glomerulus (see Table 1). The dorsal branches and varicosities were not analysed because they are usually hidden by the main axon; also they play little part in forming synapses with GI3 (cf. Blagburn and Thompson, 1990: Figs 3 and 4). L afferents were not identified as being on control or SIN sides until after the analysis was completed.

The anteriormost medial branch of L in the presence of SIN (Fig. 6C) has a surface area of 692 ± 27 μm^2 (mean ± s.e.m., n=7 animals) while in the absence of SIN (Fig. 6D) this branch has a surface area of 639 ± 56 μm^2 (n=7), a difference which, using the 2-tailed t-test, gives a probability of greater than 20% which is not therefore statistically significant (Table 1). Taking the larger, but less well defined portion of the neuron (the axon within the cercal glomerulus and all the medial, horizontal processes) shows that the surface area of L in the presence of SIN has a surface area of 5349 ± 346 μm^2 (mean ± s.e.m., n=7 animals) while in the absence of SIN (Fig. 6D) this region has a surface area of 5286 ± 318 μm^2 (n=7). Using the 2-tailed t-test gives a probability of greater than 20% showing that the difference is again not sta-

Table 1. *Measuring surface areas of specific regions of the L arborization, either in the presence or absence of SIN, fails to reveal any anatomical concomitant of synaptic competition*

	Arbor	Black branch surface area (μm^2)	shaded area plus black area (μm^2)
In **Presence** of SIN	1 R	696	4493
	7 R	536	4132
	4 L	736	5510
	5 R	729	5510
	3 R	725	5371
	11 L	695	5424
	8 R	726	7005
Mean±s.e.m.		**692±27**	**5349±346**
In **Absence** of SIN	1 L	809	5531
	9 R	760	6129
	12 L	584	5621
	5 L	472	4432
	3 L	578	3941
	11 R	474	5155
	8 L	797	6190
Mean±s.e.m.		**639±56**	**5286±318**

Inset left: the two regions of the L arborization chosen for the quantification presented in the table are indicated for afferent 1R. The arbor identities presented in the table relate to the arborizations in Fig. 6C,D, read from left to right.

tistically significant (Table 1). By both measures therefore, we conclude that the presence of SIN has no significant effect on the anatomy of L.

Discussion

Space Invader mutant affects pattern formation

The breeding data show that there is a genetic, and not environmental, basis to the production of supernumerary filiform hairs in our laboratory cockroach colonies, hence we are justified in using the term "mutant". In interpreting the results from these mutants, we are employing the basic assumption that the mutation disrupts only pattern formation, causing the production of supernumerary sensilla, but leaves other aspects of neuronal development unaffected. The supernumerary neurons (SIN, SuM and C) in these mutant animals display precisely the anatomy and connectivity of normal second instar neurons that are born at equivalent positions (Blagburn et al., 1991; Thompson et al., 1992). They do this despite the fact that they are truly supernumerary and not simply premature second instar neurons. The supernumerary cells are therefore responding to positional cues in a completely normal fashion. We are challenging the system, therefore, simply by the addition of extra neurons, thus any effects observed reflect normal developmental processes.

Anatomy of the supernumerary afferents

SIN, like L, restricts its arborization to the ventro-lateral region of the cercal glomerulus, and SuM, the rarer supernumerary medial afferent, occupies the same region of the cercal glomerulus as does the normal M (Blagburn et al., 1991). The supernumerary afferents do not concentrate their arborization in the centre of the cercal glomerulus, where presumably there would be less competition from the normal afferents, and where all the axons are capable of forming branches. In the cockroach therefore, as in the cricket (Bacon and Murphey, 1984; Murphey, 1985, 1986), it is the circumferential position of the neuron on the cercus, rather than competitive interactions, which is the major determinant of afferent location within the CNS. This powerful mechanism that targets an afferent to a particular region of neuropil seems to render it incapable of projecting to adjacent neuropil regions.

Supernumerary filiform afferents compete with normal afferents for synaptic targets

Our electrophysiological results show clearly that the synaptic input of the normal afferents to their target interneurons is reduced when a supernumerary afferent is also present. This could simply be due to a decrease in input resistance of the GI as the number of afferents increases. However, we show that the EPSPs from M are reduced proportionately more than those from L in the presence of SIN (M reduced to 50%, L reduced only to 70-80%). This suggests strongly that the reduction of EPSP size is not due to a decrease in input resistance, since this would be expected to reduce M and L synaptic potentials by the same amount. It could be postulated that the filiform afferents form synapses onto different regions of the GI3 dendrites, and that in the presence of SIN the electrotonic distance of the M synapses from the cell body is increased disproportionately. However, reconstruction of GI3 dendrites from ultrathin sections (Blagburn et al., 1985) has shown that inputs from the M axon are located on the same GI3 dendrites which, a few microns further from the cell body, go on to form contacts with the L axon.

The formal definition of "competition" requires that the protagonists interact indirectly, only affecting each other by reducing the supply of some limited resource. It is usually difficult to distinguish between a situation in which neurons are competing for a resource and one in which they are inhibiting each other directly. It is pertinent that, as mentioned above, the M to contralateral GI3 synapse suffers a greater reduction than the L - contra GI3 synapse in the presence of SIN, despite the fact that SIN and L are anatomically more closely associated. Were an inhibitory substance being released from each afferent, it would be expected that L would be more adversely affected than M by the presence of SIN.

It is noteworthy that the total excitatory synaptic input to the GI in question remains approximately the same, whether or not an extra afferent is present. This suggests that it is some aspect of the postsynaptic cell which is the limiting factor, and implies that the interneurons operate near the limit of their ability to form synapses. In this case, addition of an extra presynaptic cell would result, not in the production of new "resources", but in the redistribution of existing ones. The question of the molecular nature of these "resources" remains open and should be the subject of further study.

An additional way to study these interactions would be to perform quantal analysis. Using this approach, Shepherd and Murphey (1986) have shown that the increase in efficacy of an identified synapse which results from removal of one cercus is associated with an increase in quantal content.

Is there an anatomical basis for the competitive interaction?

Our results show that, at least at the level of resolution afforded by the light microscope, there is no anatomical concomitant of the competitive interaction between the afferents. An interesting parallel occurs in the cricket, where transplantation of a cercus into the contralateral socket induces supernumerary appendages to grow, and these bear the same identified sensory neurons as in normal cerci. The arborization of these supernumerary neurons occupy the same neuropilar territory as the normal axons but they are no smaller than normal, even though the "competition" with the cercal glomerulus is much greater (Murphey et al., 1983).

Meaningful quantification of anatomical change is a difficult task; we decided not to count varicosities in the same way as Murphey et al. (1983) had done in the cricket because in the first instar cockroach, a significant part of a filiform afferent's arborization consists of the stout axon shaft and side branches, which cannot be divided easily into varicosities but which do make large output synapses to GIs and other neurons (Blagburn et al., 1985). In addition, although varicosities appear to be sites where branches distend to

accommodate mitochondria and synaptic vesicles and output synapses are reliably formed (Blagburn, 1989), they can also be formed at other positions (Watson and Burrows, 1985). We decided, therefore, to measure the total surface area of identifiable regions of the L arborization. Whether the number of synapses formed is proportional to the surface area is unclear, although there appears to be a causal relationship between the surface area of fly photoreceptor terminals and the number of synapses which they make (Meinertzhagen, 1989). In our study, we find that there is no significant difference between the surface area of equivalent regions of the L arborisation in the presence or absence of SIN; however we cannot conclude that the number of output synapses is the same. An obvious way to resolve this question would be to quantify the synapses between an identified afferent - GI pair using the electron microscope, in the presence and absence of a supernumerary afferent.

What does this tell us about normal development?

Since the presence of a supernumerary afferent is an abnormal occurrence, it could be argued that the interactions between neurons revealed by studying these mutant animals do not necessarily take place in normal development. All the anatomical and electrophysiological evidence so far points to the interpretation that the mutant phenotype represents a duplication of a sensillum type which would normally develop later in the second instar (Blagburn et al., 1991; Thompson et al., 1992). The EPSP amplitudes of L and M onto GIs are reduced in the second instar, suggesting that the new afferents compete with the old to form synapses with the GIs (Thompson et al., 1992). If this is the case, the supernumerary afferents in the mutant animals are using exactly the same developmental mechanisms as their normal counterparts but are simply entering the competition at an earlier, embryonic, stage.

The experimental evidence obtained by ablation of L shows that the strength of the M to GI3 connection increases, albeit in the presence of SIN. This suggests that M has a greater capacity to form synapses but is partially prevented from doing so by the presence of L. This competitive interaction between L and M would be of equal importance during normal development in wild-type animals. Obviously, this experiment should ideally be carried out in the embryo before the afferents have contacted their targets, but at present this is not technically feasible.

It can be argued that the "increase in one component produced by the removal of another" is not necessarily evidence that competitive interactions occur between them during normal development (Guillery, 1988). In the case of the cricket cercal system, it has not yet proved possible to produce changes in the X afferent arborization in response to non-destructive manipulation of the contralateral cercus (Murphey and Chiba, 1990), and no experiments have been carried out using the opposite approach, that of adding sensory neurons. Our non-interventionist study of mutant animals, however, does strongly support the conclusions, derived from surgical manipulations (Murphey, 1986; Shepherd and Murphey, 1986; and this study), that competitive interactions are an important facet of neural development in insects.

Neuronal competition has been demonstrated in a range of vertebrate and invertebrate preparations and the focus of research effort is switching from describing its phenomenology to a study of its cellular and molecular mechanisms. A difficulty is that the classical preparations may comprise thousands (or millions) of neurons. The minimum, experimentally ideal, system however, is one comprising two identified competing afferents and one identified target cell. This first instar mutant cockroach has such a system.

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