Development of synapses between identified sensory neurones and giant interneurones in the cockroach *Periplaneta americana*

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**Summary**

The cereal afferent, giant interneurone pathway in *Periplaneta americana* was used as a model for synapse formation. The morphology of the two identified filiform hair sensory neurones (FHSNs) and of two giant interneurones (GI2 and GI3) was followed throughout embryogenesis by cobalt injection. The FHSN axons enter the CNS at the 45 % stage of embryogenesis, branch at 50 % and form complete arborizations by 70 %. The giant interneurones send out a primary dendrite at 45 %. Secondary branches form between 50 % and 60 % and elaboration of the branching pattern takes place until 80 % embryogenesis. At early stages the FHSN axons are within filopodial range of GI dendrites which may use these sensory processes as guidance cues.

Synapse formation between the main FHSN axon shafts and GI dendrites was investigated by injection of the latter with HRP. From 55 % to 65 % the process is initiated by desmosome–like filopodial contacts, with subsequent vesicle clustering and formation of a small synaptic density. Numbers of contacts did not significantly increase after about 70 %, but the number of synapses doubled between 65 % and 75 %, with each GI process becoming postsynaptic to two FHSN synapses and the presynaptic densities lengthening to become bars. From 75 % embryogenesis to hatching there is a further small increase in synaptic bar length.

In the first instar GI3 is postsynaptic to both FHSN axons, whereas GI2 forms very few synapses with the axon of the lateral FHSN (LFHSN). This imbalance of contacts is present throughout synaptogenesis, apart from some early filopodial contacts. GI3 forms synapses with the lateral side of the LFHSN axon from 60 % embryogenesis but these are totally absent at hatching. The growth of glia along this side of the axon during the last 30 % of development appears to be associated with degeneration of synapses in this region.

Thus, as the dendrites of the GIs grow to form a miniature version of the adult without loss of branches, there is little evidence of an initial overproduction of FHSN–GI synapses. Similarly there is no evidence that GI2 forms ‘incorrect’ synapses with the axon of LFHSN. However, GI3 contacts are removed from an inappropriate region of a correct synaptic partner, LFHSN.

**Introduction**

The problem of how neurones locate and form synapses with the correct targets is one of the central questions of neurobiology. This problem can be divided into

**Key words:** Insect development, synaptogenesis, identified neurones, *Periplaneta americana*, interneurones.
two aspects: the first concerns the means by which the growth cones of axons and their branches are able to navigate accurately to potential synaptic targets; the second concerns the way in which neurones establish synapses with the correct cells and subsequently regulate the numbers of those contacts. Description of the embryonic development of simple model systems may help to elucidate these mechanisms. In the present investigation we use the cockroach cercal afferent, giant interneurone pathway as a model for studying the morphogenesis of identified presynaptic and postsynaptic neurones. In particular we describe the stages of synaptogenesis and investigate quantitatively the changes in synapse distribution between these structurally and physiologically identified cells which take place during embryogenesis.

Studies of the behaviour of growth cones in cell culture suggest that filopodia are regularly extended and retracted (Wessels et al., 1980). By a combination of filopodial adhesion to a particular substratum and the generation of tension, the growth cone moves towards the point of attachment (Bray, 1982). Adhesion of filopodia appears to guide the growth cones of grasshopper axons in vivo (Taghert, Bastiani, Ho & Goodman, 1982) and axons growing in the peripheral and central nervous systems of Orthoptera follow specific guide-post cells and are themselves used as 'labelled pathways' by later axons (Bate, 1976; Bentley & Keshishian, 1982; Raper, Bastiani, & Goodman, 1983a, b).

The growth patterns of neuronal branches vary, from 'initially directed growth' where no inappropriate branches are formed as in the grasshopper descending contralateral movement detector (Bentley & Toroian-Raymond, 1981), cercal sensory axons (Shankland, 1981a), medial giant interneurone (Shankland & Goodman, 1982) and the cockroach giant interneurone 2 (Blagburn, Beadle & Sattelle, 1985) to cases in which transitory branches or axons are produced, such as grasshopper stretch receptor (Heathcote, 1981), dorsal unpaired median extensor tibiae (Goodman & Spitzer, 1981) & H cell (Goodman, Bate & Spitzer, 1981). Little is known about the cellular mechanisms which produce such growth patterns, although in the case of grasshopper medial giant interneurone (MGI), the presence of cercal sensory afferent axons enhances the production of small dendritic branches (Shankland, Bentley & Goodman, 1982). The spatial ordering of cricket cercal sensory axons within the terminal ganglion or within different ganglia is determined by the location of the cell bodies in the cercal array (Murphey, Bacon, Sakaguchi & Johnson, 1983; Murphey, Johnson & Sakaguchi, 1983).

The morphology of synaptogenesis has been widely studied. In vertebrates desmosome-like contacts precede synaptic vesicle clustering and presynaptic density formation (for example Rees, Bunge & Bunge, 1976; Hinds & Hinds, 1976) and similar morphological stages were seen in developing antennal lobes of the moth Manduca sexta (Tolbert, Matsumoto & Hildebrand, 1983). The sequence of events involved in synaptogenesis can be studied more effectively using identified cells. Such a model system could also provide information about the specificity of synapse formation, and about changes in the numbers and size of synapses during development.
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Cerebral sensory neurone, giant interneurone pathways are present in cockroaches (Periplaneta americana) and aspects of their anatomy (Harrow, Hue, Pelhate & Sattelle, 1980; Daley, Vardi, Appignani & Camhi, 1981), pharmacology (Sattelle, 1980; Sattelle et al. 1983) and behavioural physiology (Callec, Guillet, Pichon & Boistel, 1971; Westin, Langberg & Camhi, 1977; Camhi, Tom & Volman 1978; Ritzmann & Camhi, 1978) have been studied. For the purpose of development studies, the cockroach cerebral afferent, giant interneurone pathways offer a particular advantage over the corresponding pathways in other Orthopteroid insects. In grasshoppers and crickets the sensory axons cannot be identified without the use of intracellular tracers, however first instar P. americana possess only two filiform hair sensilla on each cercus and each neurone gives rise to an axon which can be identified in ultrathin sections by its characteristic size, position and morphology, without prior tracer injection (Blagburn & Beadle, 1982). In addition the neuronal cell bodies can be impaled with microelectrodes allowing dye injection and intracellular recording. Cobalt injection into the sensory neurones provides further evidence that the axonal morphology can be used to identify each sensory neurone (Blagburn, Beadle & Sattelle, 1984).

In previous studies the development of cholinergic sensitivity in the cell body and dendrites of giant interneurone 2 (GI2) has been described (Blagburn et al. 1985) and synapses between an identified filiform hair sensory neurone (FHSN) and GI 3 have been located (Blagburn et al. 1984). In the present study we describe the contributions of the two FHSNs to the synaptic input of GI2 and GI3 and the development of these synapses during embryogenesis.

MATERIALS AND METHODS

Oothecae projecting from female cockroaches were harvested daily and could be dated to within 24 h. Each daily collection was stored at a temperature of 30 °C. The age of embryos was expressed as a percentage of the total time to hatching (normally 31 days). Detailed descriptions of the embryos throughout embryogenesis are given elsewhere (Blagburn et al. 1985). The designation A1-A6 is used for abdominal ganglia in the first instar; A11 is used to refer to ganglionic rudiments present in the embryonic abdominal CNS. The terminal ganglion refers to the postfusion ganglion (A6) formed from rudiments A7-A11.

Newly hatched first instar nymphs or embryos of the desired age were placed in saline of the following composition: 150mM-NaCl, 3.1mM-KCl, 5.4mM-CaCl2, 5.0mM-HEPES buffer, pH 7.4 (based on Callec & Sattelle, 1973). The animals were dissected as described in an earlier paper (Blagburn et al. 1985) in order to remove the CNS which was then mounted on a microscope slide. The A6 ganglion was secured, and the connective tissue sheath was softened by brief (15 s) exposure to saline containing 1.0mg ml⁻¹ protease (Type XIV, Sigma Chemical Co.).

Isolated preparations were viewed with a Zeiss X 40 water-immersion lens, using differential interference contrast (Nomarski) optics. Electrical isolation of the objective from the body of the microscope was achieved with a Perspex insert. The somata of GI2 and GI3 were identified by their characteristic size, location and appearance. The procedure for viewing FHSNs has been described previously (Blagburn et al. 1984).

For intracellular cobalt injection, neuronal cell bodies were impaled with 50–100 MΩ micro-electrodes containing 6% hexamine cobaltic chloride (Sigma Chemical Co.). Square, positive current pulses, 0.5 s in duration at a frequency of 0.5 Hz and of 5 nA amplitude, were passed through the electrode for 3–8 min. The preparation was left in saline for 10–15 min to allow for
distribution of cobalt, followed by precipitation with (NH₄)₂S and tissue fixation with alcoholic Bouin's fixative for 30 min. Cobalt-stained cells were silver-intensified using the whole-mount Timm's procedure of Bacon & Altman (1977), dehydrated in an alcohol series, cleared, and mounted in neutral Canada Balsam. Specimens were drawn with the aid of a Zeiss drawing tube.

Giant interneurones were also labelled intracellularly for electronmicroscopical examination. Cell bodies were impaled with microelectrodes containing 4% horseradish peroxidase (HRP Type VI, Sigma Chemical Co.) with 0·3M-KCl and 0·2M-Tris buffer (pH 7·4). Current pulses of approximately 3–4nA amplitude were passed through the electrode for 2–3 min. The preparation was left in saline for 15 min, fixed in 2·5% glutaraldehyde in 0·1M-phosphate buffer (pH 7·4) containing 0·2M-sucrose for 2 h. The HRP reaction product was developed using the method described by Watson & Burrows (1981). Tissues were placed in 0·1M-Tris buffer pH 7·4 for 10 min, then in Tris buffer containing 0·5% cobalt chloride for 10 min. After washing in Tris buffer and phosphate buffer containing 0·2M-sucrose the ganglia were incubated in a medium containing 10 cm³ 0·1M-phosphate buffer pH 7·4, 4 mg ammonium chloride, 20 mg β-D-glucose, 5 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma Isopac) and 20–30 units glucose oxidase (Type V, Sigma) for 20–40 min in the dark at 37 °C. After an overnight wash in 0·1M-cacodylate buffer (pH 7·4) containing 0·2M-sucrose, tissues were osmicated for 12 h, dehydrated, embedded and sectioned horizontally at 80 nm.

RESULTS

Morphology of filiform hair sensory neurones, GI2 and GI3 in the first instar

The two first instar filiform hair sensory neurones (FHSNs) from each cercus form individually recognizable arborizations within the neuropile of the fused ganglion rudiments A₈–A₉ in the cockroach terminal abdominal ganglion, in a region similar to the cereal glomerulus of first instar grasshoppers and crickets (cf. Shankland, 1981; Bacon & Murphey, 1981). As each axon enters the neuropile from cercal nerve XI (nomenclature of Roeder, Tozian & Weiant, 1960) it exhibits one or two distinctive kinks and its diameter increases from approximately 2–3 μm to 5–8 μm. The axon arising from the lateral FHSN (Fig. 1A) is most lateral within the neuropile, and projects anteriorly along the ventral margin of the cereal glomerulus until it reaches the anterior boundary of the glomerulus which coincides approximately with the boundary between ganglion neuromeres A₉ and A₈. At intervals the axon gives rise to four or five lateral branches which curve dorsally, branching extensively around the lateral margin of the glomerulus, and five or six medial branches which project across the ganglion and recurve dorsally around the glomerulus forming many small branches.

The medial FHSN (MFHSN) gives rise to an axon (Fig. 1B) which runs medial and slightly dorsal to that of the lateral FHSN (LFHSN). It follows the latter anteriorly until the level of the A₉ commissure when it deviates approximately 45° in an anteromedial direction. The MFHSN axon also gives rise to four or five lateral and five or six medial branches whose arborization is similar to that of LFHSN. Both axons innervate similar regions of the cereal glomerulus with the exception of the anterolateral sector, from which MFHSN is largely absent as a result of the deviation in its course. The branches of both axons bear numerous swellings (1–2 μm in diameter) which probably represent synaptic boutons.

In the first instar, GI2 and GI3 (Figs 1C and 1D) display miniature versions of
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The adult branching patterns described by Harrow et al. (1980) and Daley et al. (1981). The morphology of the first instar and embryonic GI2 has been described previously (Blagburn et al. 1985). GI3 is similar in its overall morphology to GI2 (Blagburn & Beadle, 1982) possessing a transverse neurite, anteriorly directed.

Fig. 1. Drawings of silver-intensified cobalt fills of first instar filiform hair sensory neurone (FHSN) axons and giant interneurones (GI2 and 3). (A) First instar lateral FHSN (LFHSN); (B) first instar medial FHSN (MFHSN); (C) first instar GI3; (D) first instar GI2. Scale bar=50 \( \mu \text{m} \).
axon and dendrites in the contralateral cercal glomerulus and along the ipsilateral neurite. However, the soma of GI3 is somewhat larger than that of GI2 (approximately 30 µm compared to 26 µm in diameter) and is located posterior to nerve VII instead of nerve VIII. The neurite of GI3 crosses the ganglionic midline in the A_8 commissure while that of GI2 runs in the A_9 commissure. The major contralateral arborization fills the whole of the cercal glomerulus whereas that of GI2 occupies only the posterior two-thirds. GI3 does not give rise to an axon collateral branch in the A_7 neuromere.

**Growth of filiform hair sensory axons**

The FHSN axons were followed through embryogenesis using cobalt injection and electronmicroscopy. The axons enter the CNS via nerve XI at about the 45% stage of embryogenesis (Fig. 2A) and grow anteriorly. They do not fasciculate with the medial tracts of small cercal axons but take an independent course through the centre of the neuropile (Figs 2B, E). During this period of growth the axons bear numerous filopodia up to 20 µm in length. The growth cones of both axons tend to follow temporarily the path of axons in nerve X, out of the ganglion (Fig. 2A). Long filopodia are retained in this region after the growth cone has progressed anteriorly (Fig. 2E). LFHSN continues to grow anteriorly until about 60% embryogenesis when growth in this direction stops at the A_7-A_8 boundary. The MIFHSN axon grows alongside that of LFHSN until about 50-55% when at the level of the A_9 commissure it deviates in an anteromedial direction. It is not certain what environmental cues dictate this change in direction. Branches of FHSN axons appear after about 50% (Fig. 2C) and continue growing transversely, then dorsally, until 65-70% embryogenesis, when the arborizations are apparently complete (Fig. 2F). During the last 35% of embryogenesis the cercal glomerulus shortens and widens and the unbranched posterior segments of the axons undergo a reduction in length (Figs 2D, G). The number of synaptic boutons increases during this period.

**Growth of giant interneurones 2 and 3**

At 50% embryogenesis both GI2 and GI3 have sent out transverse neurites which extend to the contralateral longitudinal axon tract (Figs 3A,D). The neurite of GI3 runs through the anterior commissure of ganglion rudiment A_8, while that of GI2 runs through the commissure in A_9. Both axons turn anteriorly into the longitudinal axon tract and by 50% embryogenesis have left the terminal ganglion. At the turning points the axons give rise to posteriorly directed primary dendrites which form secondary branches between 50% and 55% embryogenesis. The small

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**Fig. 2.** Drawings of silver-intensified cobalt fills of embryonic filiform hair sensory neurone (FHSN) axons.

(A) 45% LFHSN; (B) 50% LFHSN; (C) 60% LFHSN; (D) 75% LFHSN; (E) 50% MFHSN; (F) 67% MFHSN; (G) 75% MFHSN. Percentages refer to the stage of embryogenesis. Scale bar=50 µm.
Fig. 5. (A) Two drawings of the effect of cautery on the banding pattern of *Ephestia* (redrawn from Kühn & Von Engelhardt, 1933). In both cases there is a ring of light coloured scales around the site of cautery, connected by a bridge to the nearest cross-band. (B, C, D) Attempts at simulating the effects of cautery illustrated in A. In all three panels the wing is modelled as a rectangle. Stars represent positions of three sources elucidated by Kühn & Von Engelhardt (1933; see also Nijhout, 1978, 1984b). The source levels were set at 1500 for the anterior (top) source in each panel, 1250 for the middle source and 1000 for the posterior source. The interpretation landscape is modelled as a flat plane at a value of 300 throughout. In B the open rectangle models a barrier to the diffusion of the morphogen produced by the sources. In C and D the open squares model sinks for the same morphogen. Pattern (black) is plotted where the ratio between the focal morphogen and the interpretation landscape is 0.5 ± 0.2. Modelling cautery as a barrier to diffusion does not induce the formation of a ring around the site of cautery (B). Smaller barriers have even less effect on the morphology of the banding pattern than the barrier shown in B, which is three times as wide as the size of the cauteries modelled in C and D. Modelling a cautery as a sink for morphogen yields a reasonably accurate reproduction of the experimentally obtained patterns shown in A.
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Fig. 4. The two FHSN axons can be identified in longitudinal ultrathin sections by their size, position and characteristic branching patterns. This figure contains examples of electronmicrographs of filiform hair sensory neurone, giant interneurone (LFHSN–GI3) synapses. (A) GI3 contact (asterisk) makes two dyadic synapses (arrows) with LFHSN axon. (B) High-power micrograph of synaptic bar in transverse section. The postsynaptic processes are not labelled with HRP. (C) GI3 branch (asterisk) forming output (closed arrow) and input (open arrow) synapses with unidentified neurones. (D) Coated vesicles fusing with LFHSN membrane (arrow) close to LFHSN GI3 synapse. The GI3 process is labelled (asterisk). Scale bars=0·2 μm.

dendritic branches in the neuropile ipsilateral to the cell body begin to develop in this period as does the axon collateral of GI2 in the A� ganglion rudiment. At 50%, GI3 also produces small collateral branches in this region but they disappear before 60% embryogenesis.

At this early stage the neuropile is proportionately narrower than it is after consolidation, with the result that the primary dendrites of the GIs are closer to the FHSN axons. Comparison of cobalt fills of afferents and interneurones suggests that

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Fig. 3. Drawings of silver-intensified cobalt fills of embryonic giant interneurones (GI2 and GI3).
(A) 50% GI3; (B) 65% GI3; (C) 75% GI3; (D) 50% GI2; (E) 65% GI2; (F) 75% GI2. Percentages refer to the stage of embryogenesis. Scale bar=50 μm.
all four neurones have the possibility of filopodial contact with each other since they approach to within 20\,\mu m of each other and bear many filopodia which attain this length. The arborizations of both GIs are elaborated by secondary and tertiary branching between 50\% and 80–85\% (Figs 3B,C,E & F), after which time filopodia disappear and the arborizations closely resemble those in the first instar. It is apparent that the branching patterns of the FHSNs are complete by 65–67\% whereas the GI dendrites bear filopodia until about 80\%, suggesting that they may use FHSN processes as guidance cues.

Synapses between the filiform hair sensory axons and GI2 and GI3 in the first instar

It has been shown previously that the axon of LFHSN makes numerous input synapses onto dendrites of GI3 in the first instar (Blagburn et al. 1984). The majority of the synapses between LFHSN, MFHSN and GI2 and GI3 exhibit a dyadic configuration with two postsynaptic processes, one of which belongs to the stained cell (Fig. 4A). A presynaptic bar is located between the two postsynaptic profiles, this being triangular in cross section (Fig. 4B) and 200 ± 30\,\text{nm} (n = 14) in length. On average there are two such bars at every FHSN-GI contact. Coated vesicles are commonly seen fused to the presynaptic membrane in the vicinity of such contacts (Fig. 4D) and filamentous material is present in the synaptic cleft. Previous studies of thoracic ganglionic neuropile have described similar presynaptic bars in unidentified neuronal processes (Wood, Pfenninger & Cohen 1977; Lane, Sattelle & Hufnagel, 1983) and in the latter study the fusion of coated vesicles to presynaptic membranes was thought to represent membrane retrieval.

Dendrites of GI2 and GI3 which receive synaptic input from the axon shafts of LFHSN and MFHSN were reconstructed from serial or semiserial sections (Figs 5A,B). The distribution of synapses shown in these reconstructions was found to be representative of all the other specimens examined. Dendrites of GI2 contact all sides of the MFHSN axon and approximately 66 MFHSN-GI2 synapses were counted in this reconstruction. In contrast, despite the proximity of GI2 dendrites to LFHSN, very few synaptic contacts were observed. In the reconstruction one such synapse was seen, but in most other specimens examined no synapses were observed between the axon shaft of LFHSN and dendrites of GI2. Since the whole arborizations of the FHSN axons were not reconstructed, the possibility remains that the relative contribution of LFHSN and MFHSN to the input of GI2 may differ widely in other regions of the neuropile. GI3 dendrites form numerous synaptic contacts with both MFHSN and LFHSN (approximately 20 and 85 respectively in this partial reconstruction). Although GI3 dendrites were occasionally observed in the neuropile on the lateral side of the LFHSN axon, in no case were synapses formed between them. Much of the lateral side of this axon is sheathed in two or three glial layers.

All of the synapses observed between the sensory axons and the interneurones were input synapses onto the latter. Although both GI2 and GI3 form output synapses in this region of neuropile (Fig. 4C) no such contacts were observed with
Fig. 5. Reconstructions prepared using electronmicrographs of serial ultrathin sections, showing the distributions of synapses between the lateral (LFHSN) and medial (MFHSN) filiform hair receptor axons and GI2 and GI3 in the first instar. Small FHSN branches have been omitted in order not to obscure GI dendrites. (A) Input synapses from LFHSN and MFHSN onto dendritic branches of GI3. 85 LFHSN–GI3 synapses and 20 MFHSN–GI3 synapses were reconstructed in this area of the neuropile. GI3 forms more contacts with the FHSN axons which were not reconstructed. (B) Input synapses from the axons of the LFHSN and MFHSN onto branches of GI2. One LFHSN–GI2 synapse and 66 MFHSN–GI2 synapses were seen. No other branches of GI2 contact the main FHSN axons. Scale bar=5 μm.
either MFHSN or LFHSN. However, GI-FHSN synapses may occur in other regions of the neuropile.

**Synapse formation**

The ultrastructure and distribution of developing FHSN-GI synapses were observed and the synapses formed with GI 3 by the medial side of the main LFHSN axon were analysed quantitatively. Since small numbers of individuals were sampled the values should be taken only as estimates. By the 55 % stage of embryogenesis both FHSN axons have established their main branches, whereas GI 2 and GI 3 have extended a primary dendrite and some secondary branches. The earliest contacts were seen at this stage, between filopodia of GI 2 and GI 3 and anterior branches of LFHSN and MFHSN. In later stages the GI dendrites grow outwards from the centre of the cercal glomerulus and form contacts, first with the more posterior FHSN branches (Fig. 6), then with the axon of MFHSN and finally with the axon of LFHSN. In these stages an apparent sequence of contact types can be seen, ranging from preliminary filopodial contacts on the posterior regions of the

![Fig. 6. Electronmicrograph of the neuropile at 65% embryogenesis, with anterior towards top left. The FHSN axons can be recognised at this stage on the basis of size, position and branching pattern. Part of a medial LFHSN branch (LFHSN) is shown, near its origin on the main axon. An HRP-labelled GI 3 dendrite (asterisk) is in close proximity to the branch and makes filopodial contacts (arrows) with it and with other small, unidentified neuronal profiles. Scale bar=1 μm.](image-url)
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FHSN axons, to synapses in the anterior regions and branches. By observation of this apparent sequence a tentative description of synapse formation can be made.

The first type of contact between FHSN and GIs was a simple filopodium-axon contact (Fig. 7A). The cell membranes are closely apposed over an area of 0·013 ± 0·002 μm² (n = 8) and occasionally points of membrane contact are seen. Both membranes exhibit a slight increase in electron density with filamentous material spanning the gap between them. This type of contact was not observed after 75 % embryogenesis.

Later contacts are similar in appearance with the addition of a small cluster of synaptic vesicles. At the 65 % stage approximately 70 % of this type of contact between the LFHSN axon and GI 3 exhibit a small presynaptic dense body with dimensions of approximately 30 × 30 × 30 nm (Fig. 7B) but in no case was a density observed without a small vesicle cluster. At 65 % embryogenesis LFHSN and GI 3 form approximately 0·5 ± 0·2 (n = 2) such contacts per μm² of sensory axon membrane, although they are more numerous in the anterior portion of the axon.

Maturation of synaptic contacts initially consists of an increase in the number of synaptic vesicles; evidence for two possible mechanisms was seen. At 55 % embryogenesis, 45 nm diameter vesicles were observed, enclosed in larger membranous inclusions, within FHSN axons and at maturing synaptic contacts (Fig. 7C). At 65 %, such inclusions were no longer seen but the presence of short lengths of smooth endoplasmic reticulum intermingled with synaptic vesicles suggests the in situ synthesis of vesicles (fig. 7D). Also present from this period were occasional dense-core vesicles and coated vesicles, the latter often fused with the cell membrane.

At the 75 % stage the neuropile around the FHSN axons is densely filled with cellular processes and 80 % of the LFHSN–GI 3 contacts possess a vesicle cluster, and 2 ± 0·3 (n = 7) presynaptic densities whose length has increased to 150 ± 20 nm (n = 14). The area of postsynaptic contact is also larger (0·1 ± 0·04 μm², n = 9) and the postsynaptic membrane density is more pronounced. The density of LFHSN axon–GI 3 synapses is 1·3 ± 0·1 μm⁻² (n = 3).

Few further changes in the synapses take place up to 94 % embryogenesis, apart from a small increase in the mean length of presynaptic bars to 200 ± 30 nm (n = 14) and in the area of GI 3 contacts (0·13 ± 0·03 μm², n = 10). By this stage 95 % of GI 3 contacts with LFHSN are synaptic. In the first instar the mean density of LFHSN-GI 3 synapses (1·6 ± 0·2 μm⁻², n = 3) is not significantly increased. From around 60 % embryogenesis, when the first synapse-like contacts are formed between the axon of LFHSN and dendrites of GI 3, they represent approximately 20 % of the total number of synapses formed along the axon and this proportion does not change significantly throughout embryogenesis.

In the first instar, synaptic contacts between the LFHSN axon and GI 2 are very rare. This situation exists throughout embryogenesis apart from the early stages (50–55 %), when a small number of filopodial contacts are formed with the anterior
region of LFHSN. This observation suggests that GI2 dendrites selectively form synaptic contacts with the axon of the MFHSN, not with that of LFHSN. Also, in the first instar, there are no synapses between the lateral side of the LFHSN axon
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and GI3 dendrites, despite the presence of some branches in that region of neuropile. Access to that side of the axon is prevented by glial layers. However, LFHSN–GI3 synapses form on this side of the axon from 60% embryogenesis and persist until the 95% stage, although as glia grow anteriorly along the axon some synapses disappear. Synapses are not wholly absent in this region until after hatching. At later stages of embryogenesis some vesicle clusters remain under a layer of glia, sometimes associated with degenerating postsynaptic profiles (Fig. 7E). It is not certain whether the glial growth results in this degeneration or whether the death of the postsynaptic process is necessary before the glia can grow over the synapse.

DISCUSSION

The growth patterns of the lateral and medial FHSN together with GI2 and GI3 within the terminal abdominal ganglion of Periplaneta americana resemble those of the cercal glomerular (G) afferents and medial giant interneurone (MGI) in the grasshoppers Schistocerca nitens and Schistocerca americana (Shankland, 1981b; Shankland & Goodman, 1982). The development timetables of GI2 and GI3 are similar to that of MGI, however in the grasshopper the cercal G afferents arrive in the terminal ganglion at 55–60%, whereas in the cockroach the LFHSN and MFHSN arrive earlier, at approximately 45%. The arborizations of the FHSN axons are established by 55–50% whereas those of the GIs are complete by 75–80%. Thus, in the case of the main FHSN axons, synapses are formed as a result of growth of the postsynaptic dendrites to the presynaptic cell. This is typical of the patterns of growth observed in many other models of synaptogenesis, for example: vertebrate neuromuscular junction formation in vivo (Kullberg, Lentz & Cohen, 1977) and in vitro (Nakajima, Kidokoro & Klier, 1980), vertebrate neurones in vivo (Landmesser & Pilar, 1972) and in vitro (Rees et al. 1976); moth neuromuscular junctions (Stocker & Nüesch, 1975) & Mauthner cell innervation (Jacoby & Kimmel, 1982). However, in the optic lobe of Musca domestica photoreceptor synapses are formed by growth of the postsynaptic cell to the synaptic sites (Fröhlich & Meinertzhagen, 1982). In the terminal abdominal ganglion of P. americana both types of growth may occur since postsynaptic GI dendrites grow to presynaptic FHSN axons and synaptic endings are formed on the primary dendrites of GIs.

Fig. 7. Electronmicrographs of unidentified contacts with the LFHSN axon. (A) Initial filopodial contacts (arrow) with LFHSN axon in a 55% stage embryo. Filamentous cleft material and symmetrical membrane densities are present. (B) Early synapse-like contact (arrow) between LFHSN axon and unidentified filopodium. Small vesicle cluster and presynaptic dense body are visible in the 55% stage embryo. (C) LFHSN synaptic ending with vesicles transported in membrane (arrow), at 55% embryogenesis. (D) LFHSN synaptic ending at 65% with short lengths of smooth endoplasmic reticulum (arrow). (E) Degenerating synapse on lateral side of LFHSN. Myelin figures are visible in postsynaptic processes. (arrow). A glial layer covers the synapse (asterisk). Scale bars=0.2 μm.
Since the main branches of the sensory axons are established before the GI arborizations are complete, deafferentation of embryonic cockroach GIs might be expected to have a more radical effect upon the branching pattern than it does with grasshopper MGI where the sensory afferents grow in at a later stage (Shankland et al. 1982).

Injection of HRP into GI2 and GI3 enabled an analysis to be made of their patterns of connection with the two identifiable FHSN axons. GI3 was found to receive synaptic input from both axons, in particular that of LFHSN, whereas GI2 apparently receives input almost exclusively from the MFHSN axon. The distribution of synapses seen in these restricted regions of the cells may or may not be typical of the overall synapse distribution.

If the patterns of synapse distribution seen on the axons of the FHSNs is representative of other parts of the arborizations then it can be inferred that GI3 receives excitatory cholinergic synaptic input from both LFHSN and MFHSN while GI2 receives input mainly from MFHSN. It has been shown by Dagan & Volman (1982) that in the first instar cockroach the lateral FHSNs exhibit an increased spiking frequency in response to wind from the front of the animal, while the medial FHSNs respond when the wind direction is from the rear. If the distribution of morphological synapses is a reliable indicator of the strength of the physiological connection, then GI3 will be excited by wind from all directions while GI2 will be excited by wind mainly from the rear. However, in the adult, GI3 responds mainly to wind from the front and the response of GI2 shows no directionality (Westin et al. 1977).

It may be that GI2 and GI3 undergo changes in directional sensitivity during postembryonic development, presumably as a result of changes in relative synaptic inputs as large numbers of FHSN axons enter the ganglion in later instars.

Alternatively, although synapses between the LFHSN axon and GI2 are very rare, they may occur in other regions of the cercal glomerulus. Since there are no apparent barriers preventing GI2 dendrites from approaching the LFHSN axon, the most likely explanation of such an imbalance of contacts would be the presence of different cell surface markers on the axon and branches of LFHSN, allowing adhesion of GI2 filopodia to LFHSN branches but not to its axon. To answer these questions it will be necessary to record from first instar GIs while stimulating a single FHSN.

An hypothetical sequence of synapse development was obtained by observation of FHSN-GI contacts throughout embryogenesis. This sequence is similar to those described in other studies of synaptogenesis, for example, in vertebrate neurone cultures (Rees et al. 1976); in Manduca antennal lobe (Tolbert et al. 1983). In all cases initial filopodial adhesion mediated by demosome-like contacts occurs followed by accumulation of synaptic vesicles and formation of a presynaptic membrane density. It is not certain whether some initial filopodial adhesion sites persist and become modified into synapses, or whether a temporary adhesion simply guides a dendritic growth cone to the site. The large number of immature synapse-like contacts formed by the FHSN axons with processes of small diameter that are similar to filopodia suggests that the former mechanism may be important. The
expansion in area of postsynaptic contacts seen by 75% embryogenesis may be a result of dendritic growth in the direction of filopodia anchored by synapse-like junctions. The mean number of LFHSN axon synapses at a single contact with GI 3 increases from one to two between 65% and 75% embryogenesis but there is little further increase in the density of contacts. In addition the mean length of the presynaptic densities increases during this period and the majority thereafter take the form of synaptic bars.

Counts of a defined population of synapses (those between the axon of LFHSN and GI 3 dendrites) show that there is no initial overproduction then subsequent removal of contacts, as is the case in the developing photoreceptor terminals in Musca (Fröhlich & Meinertzhagen, 1983). The number of contacts rises until 65–70% embryogenesis, and the number of synapses is approximately doubled between 65% and 75% by the expansion of postsynaptic processes and an increase in the number of synaptic bars per contact. These estimates refer to net numbers of synapses and it is possible that individual synapses may not survive for lengthy periods of time.

Despite the possibility that regional differences may exist in the density of FHSN-GI synapses, the region of neuropile around the FHSN axons remains a valuable model for investigating the degree of specificity of synapse formation during embryogenesis. There is no evidence of substantial synapse formation between the axon of LFHSN and GI 2 at any stage suggesting that, in this case, synapses are formed only between compatibly labelled cells or regions of cells. In contrast, in many vertebrate systems relatively non-specific synapse formation occurs initially, followed by synapse elimination from some target cells (Purves & Lichtman, 1980). However, the lateral side of the LFHSN axon forms synapses with GI 3 and other neurones which are later removed, either as a consequence of, or as a preliminary to, glial growth. The simplest hypothesis would be that glial processes contact the postsynaptic dendrites, somehow resulting in their degeneration and the gradual breakdown of the presynaptic side of the synapse.

Spontaneous synaptic activity can be recorded from the cell bodies of GI 2 (Blagburn et al. 1985) and GI 3 at 75% embryogenesis. This does not mean that functional synapses are absent before this stage. For example, a certain number of functional synaptic contacts may be necessary before spontaneous synaptic activity can be recorded in the soma. It has been reported that functional synaptic transmission precedes the appearance of anatomically recognizable synaptic junctions (Crain & Peterson, 1967; Landmesser & Pilar, 1972; Kullberg et al. 1977) so it is possible that FHSN-GI synaptic transmission is present before 70–75% embryogenesis. The doubling of the number of synapses seen between 65% and 75% may result in an increase in the amplitude of compound epsps in GIs allowing them to be recorded from the cell body. Electrical stimulation of FHSN axons and simultaneous recording from GIs will help to resolve these questions.

GI 2 becomes sensitive to carbamylcholine at about 40% when axonal outgrowth
commences, and the sensitivities of cell body and dendritic membranes diverge at approximately 55% embryogenesis (Blagburn et al. 1985). GI2 filopodia form contacts with other neurones as soon as the axon first sprouts, however it is not until about 50–55% embryogenesis when the dendritic tree begins to form, that filopodial contacts are established with FHSN axons. It is possible that contact with potential synaptic partners triggers the divergence of chemosensitivity in the two regions of the cell.

In the simple model system provided by cockroach filiform hair sensory neurones and giant interneurones it appears that, at least in the axonal region of the presynaptic cells, synapse formation is a relatively specific process. At no stage during its growth does GI2 form substantial numbers of ‘incorrect’ contacts with the axon of LFHSN. Similarly, no overproduction and subsequent loss of LFHSN–GI3 synapses was observed, but a rapid increase in the net numbers of contacts was seen, followed by a doubling of the number of synapses per contact. However, synapses between appropriate synaptic partners, LFHSN and GI3, which are formed in an inappropriate location, are removed towards the end of embryogenesis, possibly as a result of glial growth.

This study provides evidence that in the case of neurones whose dendrites appear to grow directly into a mature branching pattern, with no apparent loss of branches as the pathway becomes behaviourally functional, there is a large degree of specificity in the formation of synaptic contacts. Morphological studies of this type are an essential prerequisite for experiments in which manipulation of the synaptic inputs onto developing neurones is attempted.

REFERENCES

Development of synapses between identified insect neurones


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