UNUSUAL STRUCTURAL FEATURES AND ASSEMBLY OF GAP AND PLEATED SEPTATE JUNCTIONS IN EMBRYONIC COCKROACH CNS

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SUMMARY

Junctional assembly in the developing CNS in cockroach embryos has been studied during the last half of neurogenesis. Atypical linear tracts of gap junctions are found to develop between attenuated cytoplasmic glial cell processes and their overlying perineurial cells during the last third of development. During both perineurial and glial gap-junctional formation, 13 nm E face (EF) intramembrane particles (IMPs), such as are characteristic of arthropod gap junctions, are seen initially as free IMPs; these then become arranged in loose irregular clusters or alignments and finally are aggregated in plaques. P face ridges (or EF grooves), typical of tight junctions, are found on the same perineurial membrane face as assembling gap-junctional PF pits (or EF particles). Pleated septate junctions also develop between adjacent perineurial processes during the last third of embryogenesis; these form by the apparent migration of individual 8 nm PF IMPs into meandering rows, which then become aligned in numerous orderly parallel stacks. Although all these junctions occur on the same perineurial membrane face, the IMPs that form the different junctional types never appear to be confused during junctional assembly. The cues to signal the advent of these precise patterns, however, are unknown.

INTRODUCTION

The cockroach nervous system has been much studied in the adult state with regard to the intercellular associations found in the glial cell ensheathment of the ventral nerve cord (Lane & Treherne, 1970, 1972; Lane, Skaer & Swales, 1977). These glial cell–cell junctions, which are of several types, have not yet, however, been investigated during their stages of maturation in the developing embryo of this hemimetabolous insect. Studies have been made on differentiating glial gap junctions in the central nervous system (CNS) of both embryos and pupae of holometabolous insects such as the blow fly Calliphora (Lane & Swales, 1978a,b) and the moth Manduca (Lane & Swales, 1979, 1980) and also, recently, during neurogenesis in the locust Schistocerca (Swales & Lane, 1985). In the majority of these cases the mature gap junctions are characterized by circular plaques of closely clustered connexons. In a few situations, such as in the locust, atypical mature gap-junctional configurations have been reported when, within the same junctional plaque, both tightly and loosely packed connexons were found to co-exist (Swales & Lane, 1985). The connexon packing reported here in the mature gap junctions in cockroach glia is often also found

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to be pleiomorphic, as is the case when such tissues are rapidly frozen without cryoprotection (Lane & Swales, 1982a; Swales & Lane, 1983). In the present study, other unusual linear gap-junctional structures have been observed, no doubt due to the peculiar distribution of attenuated glial cell processes. The intercellular glial junctions that occur in cockroach CNS include not only these and the other more conventional gap junctions, but also, between perineurial cells, pleated septate junctions and tight junctions. The tight and gap junctions appear to form concurrently, much the same as has been found to occur in the perineurium of the spider nervous system (Lane, 1981). These junctions are found between the complex overlappings of the few but large perineurial bracelet cells and they, as in the locust (Swales & Lane, 1985), together with the narrowed intercellular cleft, are responsible for the slowing down, and ultimately the impedance to entry of foreign molecules and the formation of the blood–brain barrier (Lane & Treherne, 1972, 1980). It seems likely that the extracellular matrix between cells and perhaps also the septate junctions may play some further part in altering the speed of movement of substances through the intercellular clefts (Treherne, Schofield & Lane, 1982). The mode of assembly of these various junctional types has been examined in late cockroach embryos; the same membrane face may become differentiated into more than one junctional modification, but in every case the appropriate intramembrane particles become incorporated into each of the different junctional types.

MATERIALS AND METHODS

The embryos used in these experiments were of the cockroach, Periplaneta americana, maintained in the laboratory at 26 °C; under these conditions the eggs took 37 days after laying to differentiate and proceed to the hatching stage. Embryos were removed from egg cases for examination at intervals from day 4 onwards as well as after hatching. In the early stages the embryos, having been carefully dissected out, were opened by a lateral incision to reveal the developing ventral nerve cord, and fixation was carried out on the whole nerve cord in situ. These preparations were embedded for thin-sectioning in Araldite. In later stages, when the nerve cord was larger, it was possible to dissect it out in its entirety. It was on such isolated nerve cords that freeze-fracture was possible. The freeze-fracture results reported in this paper were carried out on embryos taken at daily intervals over the last third of embryogenesis, that is, day 24 through to day 37. Between two and five embryos were examined at each of these stages and 13 different insects after hatching.

Ventral nerve cords were dissected out in cockroach Ringer, the composition of which was as follows; trehalose, 5 mm; NaCl, 157 mm; MgCl₂, 2 mm; KOH, 3·0 mm; Hepes, 8·6 mm; CaCl₂, 2 mm. The tissues were fixed for half to 1 h at room temperature in 2·5 % glutaraldehyde in 0·05 m-phosphate buffer (pH 7·4) plus 6 % sucrose, followed by three washes in phosphate buffer plus 6 % sucrose. This was followed by cryoprotection with 20 % glycerol in phosphate-buffered sucrose for 30 min at room temperature. The tissue was mounted in yeast and sandwiched between double copper holders; these were frozen in liquid Freon 22 cooled by liquid nitrogen. Complementary replicas were prepared using a Balzers (BA 360M) freeze-fracturing device at −100°C with a pressure of 2 × 10⁻⁶ Torr (1 Torr = 133·3 Pa), followed by shadowing with tungsten–tantalum and backing with carbon. After admission of air, while still on the cold table of the fracturing unit, the replicas were coated with a drop of celloidin. Holders were removed from the device and the celloidin was allowed to dry before the complementary replicas and tissue were removed from the holders and cleaned as intact replicas with sodium hypochlorite. The celloidin was removed by submerging the mounted replicas in amyl acetate and the cleaned replicas were then examined in a Philips EM 300 at 60 or 80 kV. Micrographs are mounted with the shadow coming from the bottom or side.
Junctions in developing insect CNS

RESULTS

In the developing cockroach nervous system, as the embryo matures, expanses of the perineurial bracelet cell membrane are found to have attenuated finger-like projections of the underlying glial cells running across their surface (Figs 1, 3). This can be seen clearly in complementary replicas on both of the membrane faces. When the overlying perineurial cell PF is revealed by the fracture plane, the regions under the finger-like glial cell processes are seen to be nearly continuous arrays of aggregated PF pits (Figs 1, 2; inset, Fig. 2). This is the typical PF appearance of arthropod gap junctions, except that they are normally macular or plaque-like in outline and do not exhibit such striking linear configurations. On the E face, the overlying perineurial cell membrane expanse reveals linear arrays of complementary intramembrane particles (IMPs) or connexons (inset, Fig. 3). These can be observed to correspond to the regions where the finger-like processes of the glial cells are found, as the plane of fracture occasionally cleaves down from the EF into the outer membranes of the finger-like processes (Fig. 3). It can be seen that the gap junctions coincide with the distribution of the glial processes so that where their finger-like processes are apposed to the overlying perineurial cell membrane there is nearly always an extensive gap-junctional contact.

In the earlier embryos examined in thin sections, no evidence for junctions was observed. Assembly of the gap junctions in the perineurial and glial cells of the cockroach appears to occur toward the last half or third of embryogenesis, starting around day 24. The stages observed begin with individual putative connexons scattered over the presumptive gap-junctional membrane face (Fig. 4); these were considered to be gap-junctional due to their size (13 nm) and fracturing characteristics (E face). These IMPs become clustered into aggregates or alignments of two to six particles (Fig. 5); further clustering of the connexons occurs by day 32 (Figs 6, 7) and finally, by hatching, closely packed plaques are found (inset, Fig. 7). The timing of these stages is variable and it seems that the assembly rate may vary from glial cell to glial cell or from organism to organism. Various stages in the formation of one junctional type may occur on the same or nearby membrane face; since junctions appear to form relatively rapidly, individual junctions seem to be assembled at various intervals over the last third of embryogenesis. Those in the perineurium tend to mature rather later than those between the underlying glial cells.

Figs 1, 2. Replicas of bracelet cell membrane P faces (PF), at day 32, showing the underlying glial cell processes, which branch and run over the membrane and which, when fractured away, reveal tracts of clustered PF pits (arrows). High power (Fig. 2) shows that these clustered PF pits are characteristic of gap junctions (at arrows), with occasional EF connexons adhering to the PF. Inset: another such area where the cell process (cp) has been cleaved away. Fig. 1, × 28 900; Fig. 2, × 57 400; inset, × 66 000.

Fig. 3. Replica of the E face (EF) of a bracelet cell membrane at 32 days, revealing the linear tracts of EF particles of gap junctions (arrows), which overlie the numerous attenuated glial cell processes. These have been fractured away to reveal their widespread branching distribution. Inset: a comparable area from a 25-day embryo. × 32 500; inset, × 57 440.
Figs 1–2. For legend see p. 271
Fig. 3. For legend see p.271
Although there is a strong tendency for the gap junctions in the hatchling CNS to be in the clustered state, occasionally apparent junctions are encountered that exhibit a very loose state of packing (Figs 8, 9) or consist of scattered EF particles, which could be gap-junctional, with little organization into maculae at all. This may represent junctional turnover as described earlier in mature arthropod tissues (Lane, 1978).

Towards the end of development of the CNS, from day 31 onwards, some perineurial cell gap junctions are found in the last stages of assembly; these have been studied in complementary replicas. These complementary replicas were invaluable in finding and observing the membrane half leaflets containing the PF ridges around the gap-junctional EF pits; this membrane half tends to be fractured away in normal replicas. The complementary replicas were not used for matching individual structural components. PF pits (Fig. 10) or complementary EF particles (Fig. 11) are found, singly or in short linear arrays, scattered over the membrane face. Around the periphery of these forming gap junctions are found short rod-like structures composed of IMPs aligned in ridges on the PF (Fig. 10) or complementary grooves in the EF (Fig. 11). The putative connexons gradually cluster into macular plaques seen in the

Figs 4–7. These replicas show stages during the last third of development when the gap-junctional EF connexons are initially scattered freely on the presumptive junctional membrane face (Fig. 4) at 25 days, and then become aligned in short rows of aggregates (Fig. 5), which cluster (Fig. 6) into larger plaques at day 31 (Fig. 7) that are ultimately, by 32 days, macular and fairly regular in outline (inset, Fig. 7). Fig. 4, x 40 100; Fig. 5, x 40 500; Fig. 6, x 40 300; Fig. 7, x 34 700; inset, x 45 000.

Figs 8, 9. These micrographs demonstrate the appearance of a, supposedly, formed gap junction in mature tissues from adult cockroach. These are perineurial cell gap junctions, which are either very loosely packed in plaques (Fig. 8) or so loose as to lose their plaque-like configuration (Fig. 9). sj, septate junction. Fig. 8, x 50 700; Fig. 9, x 26 200.

Figs 10–13. These figures are taken from complementary replicas and show late stages in the formation of perineurial bracelet cell gap junctions at 31 days, towards the end of development. Short P face ridges (arrows), possibly representing forming tight junctions, are found on the periphery of the developing gap junctions in PF preparations (Figs 10, 12), and appear as complementary grooves in the EF (arrows in Fig. 11). Gap junctions (gj) are characterized by PF pits (Fig. 12) or as complementary EF particles (Fig. 11), which appear to be initially in small clusters (Fig. 10) and then become part of larger irregular aggregates (Figs 11, 12). The PF particles assume a more continuous ridge like-structure (arrows, Fig. 13) towards the time of hatching. Fig. 10, X 44 200; Fig. 11, X 32 200; Fig. 12, X 60 800; Fig. 13, X 60 400.

Figs 14–17. Replicas of perineurial cell membranes between day 24 to day 32 of embryonic development, and at hatching (Fig. 17), showing various stages in the assembly of pleated septate junctions. The junctional particles are on the P face and become aligned into individual rows (arrows) (Fig. 14), which meander over the presumptive junctional membrane (Fig. 15) at 24 days, before becoming aligned into more regular tracts (Fig. 16) at 31 days, lie parallel to one another (Fig. 17) by 32 days, exhibiting at fracture transition points EF grooves complementary to the PF IMP rows (inset, Fig. 17). In more mature tissues, gap junctions (gj in Fig. 17) may lie in intimate spatial association with the septate junctions (sj). Cross striations in the cleft between the EF and PF in septate junctions (inset, Fig. 17) may represent some component of the septal ribbons, although they could be due to plastic deformation. Fig. 14, X 60 400; Fig. 15, X 60 400; Fig. 16, X 41 800; Fig. 17, X 45 500; inset, X 68 600.
Figs 10–13. For legend see p.275
Figs 14–17. For legend see p.275
PF as aggregates of pits (Fig. 12) with PF ridges still aligned at their periphery. In some cases these may become the ridge-like tight junctions, which appear to form the permeability barrier in the CNS, in that more extensive perineurial ridges are found closer to hatching (Fig. 13).

At the same time as the gap and tight junctions are forming, pleated septate junctions arise between the perineurial cells so that in the hatchling they may lie side by side (Fig. 17). At first, the septate structure is revealed in freeze-fracture by only a few meandering rows of separated 8–10 nm IMPs (Fig. 14). With time, the numbers of these increase (Fig. 15), so that many rows come to lie side by side (Fig. 16). By these stages the complementary rows of EF pits can be seen clearly at the PF–EF fracture face transitions (inset, Fig. 17). At the time of hatching, these aligned rows of P face IMPs are highly ordered (Fig. 17), with gap junctions intercalated between their undulating IMP tracts (Fig. 17). Examination of the cleft between PF and EF in the junctional region (inset, Fig. 17) shows cross striations, which could represent some component of the intercellular septal ribbons thought to insert into the membrane at the junctional IMP sites (Flower & Filshie, 1975; Lane & Skaer, 1980).

DISCUSSION

The unusual linear tracts of gap junctions between the cockroach perineurial bracelet cell membrane expanses and the whole surface of the attenuated underlying glial cell processes have not been observed before in embryonic or adult insect CNS, including locust (Swales & Lane, 1985), blowfly (Lane & Swales, 1978a, b) or moth (Lane & Swales, 1979; 1980). They would, clearly, be identifiable only in freeze-fracture replicas and particularly by examination of complementary replicas, which explains why they were not described earlier in thin-section studies of cockroach perineurium (Maddrell & Treherne, 1967; Lane & Treherne, 1969, 1970, 1972). The significance of this unusual and extensive distribution is not clear; it may be that, in the mature CNS, particularly close coupling is required between bracelet cells and the underlying glial cell processes; this does not occur in the embryo until about day 24, the time of the formation of the blood–brain barrier. Essential integration of perineurial and glial activities may be mediated by ionic and/or metabolic exchanges or by transport of regulatory molecules via the gap junctional channels.

These stages in the formation of the cockroach embryonic glial and perineurial gap junctions are very similar to those observed elsewhere in the hemimetabolous locust glial cells (Swales & Lane, 1985) and in both dipteran and lepidopteran holometabolous insects (Lane & Swales, 1978a, 1979). The observation that the underlying glial–glial gap junctions form before the ensheathing perineurial–perineurial and perineurial–glial ones is compatible with studies on dye injection, which show that, in locust embryos, Lucifer yellow spreads rapidly from glial cell to glial cell during the whole second half of embryogenesis (M. Bate, personal communication). The intimate spatial association seen here between tight-junctional and gap-junctional assembly is akin to that reported earlier in perineurial glial cells of the spider CNS (Lane & Chandler, 1980; Lane, 1981), and in vertebrate systems under-
going developmental changes (Decker, 1981), although the tight junctions in the cockroach are far less complex.

Normally the connexons in mature gap-junctional plaques are relatively closely packed. Since some of the gap-junctional particles in these hatching or adult cockroach tissues are found to be loosely clustered, this may mean that they are not truly gap-junctional in nature, or that junctional turnover associated with cell turnover (see Lane, 1978, 1981) is occurring, these being the junctions that are currently breaking up or reaggregating. Their state of packing could, on the other hand, actually reflect the state of coupling, since it is unlikely to represent an artefact of fixation, all the different stages in aggregation being found after the same treatment. In other systems, tightly packed connexons have been held to signify that gap junctions are uncoupled (Peracchia & Dulhunty, 1976). However, very loose arrays of connexons have also been interpreted to signify cell uncoupling; this has been found to occur during cell dissociation with consequent disaggregation of gap-junctional particle clusters at insect metamorphosis (Lane & Swales, 1980) or as a result of some physiological change, perhaps initiated by anoxia (Lee, Cran & Lane, 1982). Such a pleiomorphy in gap junctions is also observed in cockroach glia after fast freezing (Lane & Swales, 1982a; Swales & Lane, 1983), which have, of course, been neither fixed nor cryoprotected; a similar pleiomorphy has been reported in rapidly frozen mammalian tissues (Raviola, Goodenough & Raviola, 1980) and in those of crustacea (van Deurs, Dantzer & Bresciani, 1982) and molluscs (Hanna et al. 1981). Each individual gap junction may be in a different physiological state, as indicated by the degree of closeness or looseness of packing exhibited; alternatively, the packing may have nothing whatsoever to do with the physiological condition with regard to coupling.

The embryonic assembly of the septate junctions described here parallels that observed in locust CNS and lepidopteran gut, with IMPS gradually taking up aligned positions into rows on presumptive junctional membrane (Lane & Swales, 1982b). The same has also been seen in regenerating Hydra (Wood & Kuda, 1980), except that there it was considered that smooth septate or continuous junctions were an intermediate stage in the development of pleated septate junctions. This seems unlikely to be so as these two junctional types are very different in tracer-stained section; interseptal columns, characteristic of the first of these seem not to be present in hydra preparations (Filshie & Flower, 1977). Moreover, hydra junctions do not exhibit all the characteristics of continuous junctions in unfixed, rapidly frozen tissues (Kachar, Christakis, Reese & Lane, 1883).

All three junctional types—gap, tight and pleated septate—are able to form concomitantly on the same membrane face, without apparently interfering in the assembly of the others. Clearly the glial membranes in insect CNS have a considerable organizational capacity to be capable of this feat of differentiation. The cues and regulatory messages that must determine these complex and precise patterns of membrane modification are as yet unknown, as are the signals that enable each cell to recognize the adjacent cell membrane with which it is forming one or more junctional association.

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REFERENCES


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