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Neurogenesis in the insect enteric nervous system: generation of premigratory neurons from an epithelial placode

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Summary

The enteric plexus (EP) is a major division of the enteric nervous system (ENS) in the moth *Manduca sexta* and contains a dispersed population of about 360 bipolar neurons, the EP cells. Previously we showed that embryonic EP cells achieve their mature distributions by extensive migration along the gut surface and then display position-specific phenotypes. We now demonstrate that the entire EP cell population is generated from an ectodermal placode that invaginates from the embryonic foregut. Individual EP cells become postmitotic just as they leave the epithelium, but their terminal differentiation is subsequently delayed until after their migratory dispersal. Clonal analysis by injection of lineage-tracing dyes has shown that the EP cell population is derived from a large number of placodal cells, each of which contributes a limited number of neurons to the ENS. Placodally derived clones produce neurons exclusively, while clones arising from cells adjacent to the placode are incorporated into the gut epithelium. These results indicate that neurogenesis in the insect ENS involves a developmental strategy that is distinct from that seen in the insect CNS and which resembles the generation of certain cell classes in the vertebrate nervous system.

Key words: enteric nervous system, placode, neurogenesis, *Manduca sexta*.

Introduction

Fundamental to the formation of the nervous system is the program of neurogenesis by which cells are generated or recruited into the developmental pathway leading to neuronal differentiation. Recent advances in the clonal analysis of the vertebrate nervous system have revealed a variety of strategies by which different cell types are produced. For example, the separation of neural from non-neural lineages can occur relatively early in development, as has been described in the cerebral cortex (Luskin *et al.* 1988; Price and Thurlow, 1988), or may not be apparent until the time of terminal mitosis, as indicated by studies of the developing retina (Turner and Cepko, 1987; Wetts and Fraser, 1988; Holt *et al.* 1988). Similarly, the commitment of vertebrate neurons to specific phenotypes can vary with respect to other phases of their differentiation. Many of the transmitter phenotypes in neural crest-derived neurons are regulated by position-specific cues encountered only after their dispersal (Patterson, 1978; Gershon and Rothman, 1984; Adler and Black, 1986), whereas the expression of peptidergic phenotypes in neurons destined to populate the hypothalamus can commence even before the cells are integrated into the CNS (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al.* 1989).

In insect systems, two predominant strategies for neurogenesis have been described. Within the developing CNS, the majority of neurons derive from identifiable stem cells, which produce characteristic sets of progeny *via* stereotyped cell lineages (Bate, 1978; Thomas *et al.* 1984). Likewise, in the PNS, an invariant lineage is thought to generate the cells that form each sensory unit, so that the sensory neurons, support cells, and epidermal structural elements appear in an orderly sequence (Lawrence, 1966; Bodmer *et al.* 1989). Clonal analysis of the developing retina in *Drosophila* has revealed a second neurogenic strategy, however, in which stereotyped lineage relationships do not play a role in the generation of photoreceptor neurons. Rather, retinal cell fate appears determined by a series of inductive interactions that regulate the progressive differentiation of identifiable phenotypes (Ready *et al.* 1976; Hafen *et al.* 1987; Tomlinson *et al.* 1988). Despite these initial differences, subsequent developmental events throughout the insect nervous system appear similar: embryonic neurons in both the central ganglia and the retina typically differentiate soon after their

terminal mitoses, with little or no dispersal from their site of origin. In this regard, neuronal development in insects appears less complicated than in many vertebrate embryos, where extensive phases of cellular migration often reorganize the spatial relationships among developing neurons and precede the expression of mature phenotypes (Weston, 1970; Sidman and Rakic, 1973; Le Douarin, 1982).

Recently we have characterized the differentiation of the insect enteric nervous system (ENS; Copenhaver and Taghert, 1989*a*,*b*), a major division of the peripheral nervous system that supplies innervation to the gut with only limited input from higher neural centers. It includes several small peripheral ganglia, as well as a more dispersed population of cells within the major nerves of the ENS that run superficially along the alimentary tract. In particular, we have focussed on the formation of the major nerve plexus of the ENS, the *enteric plexus*, during embryonic development in the moth *Manduca*. This plexus spans much of the foregut and midgut and contains approximately 360 bipolar neurons (the EP cells) that are distributed along the plexus nerves of the gut surface. The EP cells exhibit diverse morphological and biochemical phenotypes that are expressed in a position-specific manner (Copenhaver and Taghert, 1989*a*). In contrast to the pattern of differentiation observed in the insect CNS and retina, the enteric plexus exhibits many of the developmental features more commonly attributed to the neural crest and placodes of vertebrates: following their initial generation, for example, the immature EP cells become progressively dispersed by active migration along divergent pathways (Copenhaver and Taghert, 1989*b*). Unlike many central neurons in insects, the EP cells do not become uniquely identifiable but can be categorized into several distinct subtypes within the different domains of the plexus. Moreover, the expression of many of their mature characteristics is significantly delayed until after the migratory phase is complete.

To clarify the nature of the developmental program giving rise to this class of insect neurons, we have now characterized the origins of the enteric plexus and have deduced the mitotic patterns by which the EP cell population is generated. We report that all of the EP cells are derived from a common epithelial placode, arising from a defined region in the dorsal foregut surface at 30 % of embryogenesis. Within this placode, a large number of mitotically active cells serve as progenitors for the EP cells, each giving rise to a small clone of 1–8 cells that assume exclusively neuronal phenotypes. Their progeny become post-mitotic as they leave the epithelial layer, forming a discrete population of pre-migratory neurons on foregut surface. We interpret these results to indicate that the development of the EP neurons of the gut represents a third general strategy for neurogenesis in the insects.

Materials and methods

Embryonic staging and immunocytochemistry

Animal rearing and collection were performed as previously described (Copenhaver and Taghert, 1989*a*). Synchronous egg batches from a laboratory colony of *Manduca sexta* were collected in 1 h intervals as needed and incubated at 25°C; under these conditions, embryogenesis is complete in about 100 h (approximately 1 % development per h). Embryonic development could be monitored by reference to established schedules of external and internal markers (Dorn *et al.* 1987; Copenhaver and Taghert, 1989*a*). To visualize the developing ENS, staged embryos were dissected under physiological saline (modified Weever's: KCl, 34 mM; NaCl, 7 mM; $MgCl_2$, 8 mM; $CaCl_2$, 14 mM; Dextrose, 173 mM; $KHCO_3$, 1.25 mM; KH_2PO_4, 1.25 mM; pH 6.2; after Levine and Truman, 1985), restrained in a shallow Sylgard (Dow Corning) chamber, and monitored using a 50× water immersion lens and Nomarski optics. Animals prepared in this manner were also fixed for 1 h in 2 % paraformaldehyde in phosphate-buffered saline (PBS) and processed for whole-mount immunohistochemistry using the monoclonal antibody TN-1 (diluted 1:20 000 in PBS with 0.1 % Triton-X 100, 0.1 % NaN_3, and 3 % normal horse serum; Copenhaver and Taghert, 1989*b*) and visualized using the avidin–biotin–HRP system of Vector Laboratories. Stained preparations were mounted in 90 % glycerol with 0.1 % p-phenylenediamine (Caudy and Bentley, 1986) and photographed or drawn by *camera lucida* at 650× magnification.

Post-embryonic larvae were anesthetized by immersion in water and dissected under saline to expose the ENS. After a 5 min fixation in 2 % paraformaldehyde to preserve gut morphology, the alimentary tract was then further dissected and flattened onto the surface of a Sylgard-coated culture dish, followed by fixation for 1–2 h. The preparations were then briefly dehydrated with chilled ethanol (5 min rinses with incremental concentrations from 10–80 % before rehydration) to enhance tissue permeability, followed by whole-mount immunohistochemical staining as already described. Rabbit antisera were raised against commercially synthesized Phe-Met-Arg-Phe-NH_2 (FMRFamide; Sigma) that had been conjugated to thyroglobulin or bovine serum albumin. They were applied to the tissue at concentrations of 1:3000–1:5000. Immunoreactive staining was blocked by liquid-phase preabsorption of the antisera with the conjugated antigen. It should be noted, however, that the insect nervous system probably does not produce the pentapeptide form of FMRFamide but instead expresses a family of amino-terminally extended forms of this peptide (Nambu *et al.* 1988; Schneider and Taghert, 1988).

Incorporation and detection of BrdU

Staged embryos were selected at times throughout the period of ENS formation (25–85 % of development) and dissected as described above, using a modified insect culture medium: 50 % Schneider's *Drosophila* medium, 40 % Eagle's basic salts, 9.9 % heat-inactivated fetal calf serum, 0.09 % penicillin–streptomycin, 0.01 % insulin, supplemented with *Manduca* haemolymph (after Chen and Levi-Montalcini, 1969; and Seecof *et al.* 1971). Animals were then transferred to medium containing 15–150 $\mu g\,ml^{-1}$ of 5-bromo-2′deoxyuridine (BrdU; Sigma) and incubated for 2 h at room temperature with gentle agitation. Similar results were obtained with all concentrations of BrdU, although the highest concentrations induced abnormal cellular morphologies that became increasingly apparent with extended incubation periods.

Following a brief rinse with fresh culture medium, the preparations were exposed to Carnoy's fixative for 15 min, rinsed in PBS plus 0.3 % Triton-X 100, and incubated in a solution of 2 N HCl for 30 min. The tissue was then rinsed to remove the acid and transferred to PBS plus 0.3 % Triton,

0.1% NaN_3, and 10% normal horse serum for 1 h (after Truman and Bate, 1988). Whole-mount immunohistochemistry was subsequently performed as already described, using a monoclonal antibody to BrdU (Becton-Dickinson; at concentrations of 1:30–1:50). For some preparations, the enteric plexus was subsequently counterstained with the monoclonal antibody TN-1, using the avidin–biotin–HRP protocol in the presence of NiCl, or using the avidin–biotin–alkaline phosphatase procedure (Vector Laboratories) to distinguish the two immunohistochemical reactions.

Clonal analysis of cell lineages

Staged embryos were isolated in sterile culture medium and individual cells within the developing ENS were impaled with a glass microelectrode (80–100 megaohms) containing one of a variety of fluorescent lineage-tracing dyes from Molecular Probes (Gimlich and Braun, 1985): lysinated fluorescein dextran (LFD; 10×10^3 M_r, lot 7A) and lysinated tetramethylrhodamine dextran (LRD; 10×10^3 M_r, lot 8A; and 40×10^3 M_r, lot 7A) were employed with similar results. Electrodes were filled at the tip with dye concentrations of 100–200 mg ml^{-1} and backfilled with 1.2 M LiCl (after Wetts and Fraser, 1988). Cells were iontophoresed with depolarizing current pulses (100 msec, 2 nA) at 9 Hz for 15–30 s. Animals were then rinsed thoroughly with sterile culture medium and incubated in a humidified chamber at 28°C for 24 h. They were subsequently dissected and fixed in 2% paraformaldehyde for 1 h. Preparations were usually counterstained immunohistochemically with TN-1, as described above, using a fluorescent secondary antibody to complement the iontophoresed fluorochrome (e.g. fluorescein-conjugated antibodies were used for preparations injected with LRD). The labelled descendant cells were then analyzed and photographed in whole-mount using a Zeiss Axioplan epifluorescence microscope equipped with the appropriate filter sets for the different fluorochromes.

Results

Developmental origins of the EP cells

The enteric plexus spans much of the foregut and midgut and contains approximately 360 bipolar neurons (the EP cells) that are distributed along the plexus nerves of the outer gut surface (Fig. 1). Unlike many central neurons in the moth, the EP cells are not uniquely identifiable but can be categorized into several distinct subtypes that are interspersed within the plexus (e.g. Fig. 1B). For example, one EP cell class expresses substances that are related to the molluscan peptide FMRFamide (Price and Greenberg, 1977) and innervate the midgut musculature (Fig. 1C). Previously we showed that by 40% of embryogenesis, the entire EP cell population is already present in the form of a coherent, pre-migratory packet of cells on the dorsal foregut surface (Copenhaver and Taghert, 1989*b*). We therefore traced the origins of this cell group throughout the preceding stages of development both by direct observation of staged embryos (viewed under Nomarski optics) and by immunohistochemical staining with the monoclonal antibody TN-1 (Taghert *et al.* 1986). TN-1 recognizes a membrane-bound epitope expressed by cells of the developing enteric plexus (Copenhaver and Taghert, 1989*b*) as well as by several other neural and non-neural tissues in *Manduca* (Carr and Taghert, 1988, 1989), and can be used to differentiate the developing EP cells from the surrounding layers of gut epithelium.

The earliest indication of the EP cell group occurred at 30% of embryogenesis, in the form of a discrete placode that formed in the protruding dorsal lip of the foregut (Fig. 2A); at this stage, the foregut was an epithelial tube that had invaginated from the anterior ectoderm. As the cells of the placode became increasingly columnar, they could be distinguished both morphologically and when stained with the TN-1 antibody (Figs 2A, 3). The mid-dorsal position of the placode coincided with the more anterior neurogenic zones of the ENS (also TN-1 positive, Fig. 3B), which had previously developed within the foregut epithelium and which ultimately gave rise to the anterior enteric ganglia (Copenhaver and Taghert, in preparation).

Over the next 10% of embryogenesis, the cells of the placode participated in two coordinate processes (Figs 2A, 3A–F): individual columnar cells rounded out of the epithelial layer and were extruded onto the outer foregut surface, while at the same time, the placode itself underwent a progressive outpocketing from the basal surface of the gut epithelium into the body cavity – an epithelial transformation defined as an *invagination* (Fristrom, 1988). As this sequence continued, the boundaries of the placode became increasingly distinct from the adjacent cell layers (Fig. 3B, C). Concentric folding of the epithelium gradually brought the ventrolateral margins of the placode into apposition (Fig. 3D). Finally, as the last of the presumptive neurons rounded out of the placode (40%), the remaining epithelial layers fused to re-establish the continuity of the foregut surface, posterior to the newly formed cell packet (Fig. 3E, F, arrowheads).

Patterns of DNA replication in the neurogenic placode

To characterize the mitotic relationships within the gut placode and during subsequent phases of differentiation, we incubated staged embryos for 2 h periods (approximately 2% of development) with the thymidine analog, 5-Bromo-2′-deoxyuridine (BrdU). BrdU is readily incorporated into the DNA of cells in S-phase of their mitotic cycle, and when visualized with an anti-BrdU antibody (Gratzner, 1982), it can serve as a marker for the patterns of DNA replication in embryonic tissue (Truman and Bate, 1988; Bodmer *et al.* 1989). When animals were labelled with BrdU at the onset of placode formation (around 30% of development), a uniform pattern of staining was observed throughout the foregut (Fig. 3G). DNA replication was detectable in both placodal and non-placodal domains of the gut epithelium, labelling a regular but unordered distribution of cells. Once placode invagination commenced, however, BrdU incubations revealed a discontinuity in the pattern of labelled nuclei: actively replicating cells were now seen primarily within the columnar portions of the placode, while cells that were rounding out the epithelium were labelled only infrequently (Fig. 3H). This distinction became increasingly obvious in subsequent developmental stages

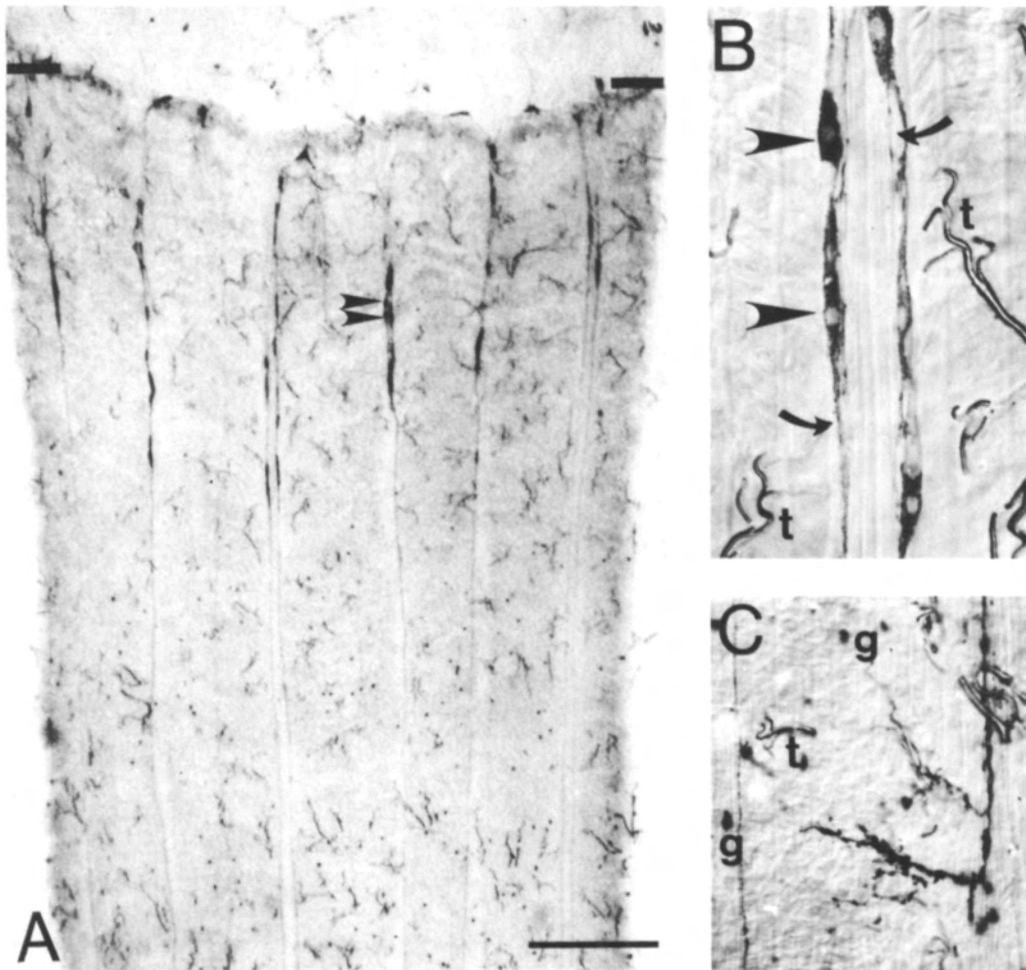


Fig. 1. FMRFamide-immunoreactive EP cells within the enteric plexus. (A) Overview of the mature enteric plexus near the foregut-midgut boundary (black hashmarks). In all figures, anterior is towards the top of the page. The gut of a post-embryonic (3rd instar) larva was cut mid-ventrally and flattened to reveal the eight major longitudinal muscle bands of the midgut along which the FMRFamide-positive EP cells (e.g. black arrowheads) are distributed. (B) Higher magnification of the same cells indicated by the arrowheads in A. Small arrows indicate some of the unlabeled EP cells that are interspersed along the muscle bands. (C) Immunoreactive processes from the EP cells projecting onto the interband midgut musculature, several hundred microns posterior to the EP cell bodies. Also visible in C are immunoreactive gut endocrine cells (g) that arise within the midgut epithelium and are unrelated to the EP neurons; t, non-immunoreactive (air-filled) trachea. Scale: 200 μm in A, 50 μm in B and C.

(Fig. 3H–K), with synthetically active cells occurring only in the lateral epithelial folds at the placode margins.

Concurrent with this gradual redistribution of BrdU labelling, the total number of stained placodal cells also changed in a consistent manner as development proceeded (Fig. 4). During the initial phases of EP cell proliferation (30–34 %), there was an increase in the number of labelled profiles occurring within the boundaries of the placode. Subsequently, this number steadily declined, as more of the cells rounded out of the epithelium. By 40 % of development, the EP cell packet was completely devoid of labelled nuclei (Figs 2B, 4), while much of the underlying foregut epithelium remained heavily labelled. In contrast, cells of the central nervous system and those derived from the more anterior neurogenic zones of the ENS continued to exhibit cycles of DNA replication (Fig. 3L arrow, and unpublished observations). The proliferation of these anterior zones will be described in a subsequent report. Incubation with BrdU during later stages of embryogenesis (up to 90 % of development) failed to reveal any additional labelling of neurons in the enteric plexus (not shown), indicating that they did not re-initiate the mitotic cycle throughout their subsequent phases of migration and differentiation. Thus in contrast to the patterns of neurogenesis seen in the insect CNS, the EP cells are generated *via* rapid proliferation from an epithelial placode, a process that gives rise to a coherent population of post-mitotic, pre-migratory neurons.

Cell lineage analysis of the neurogenic placode

The results of the foregoing experiments indicated that the generation of the EP cells occurred within a discrete

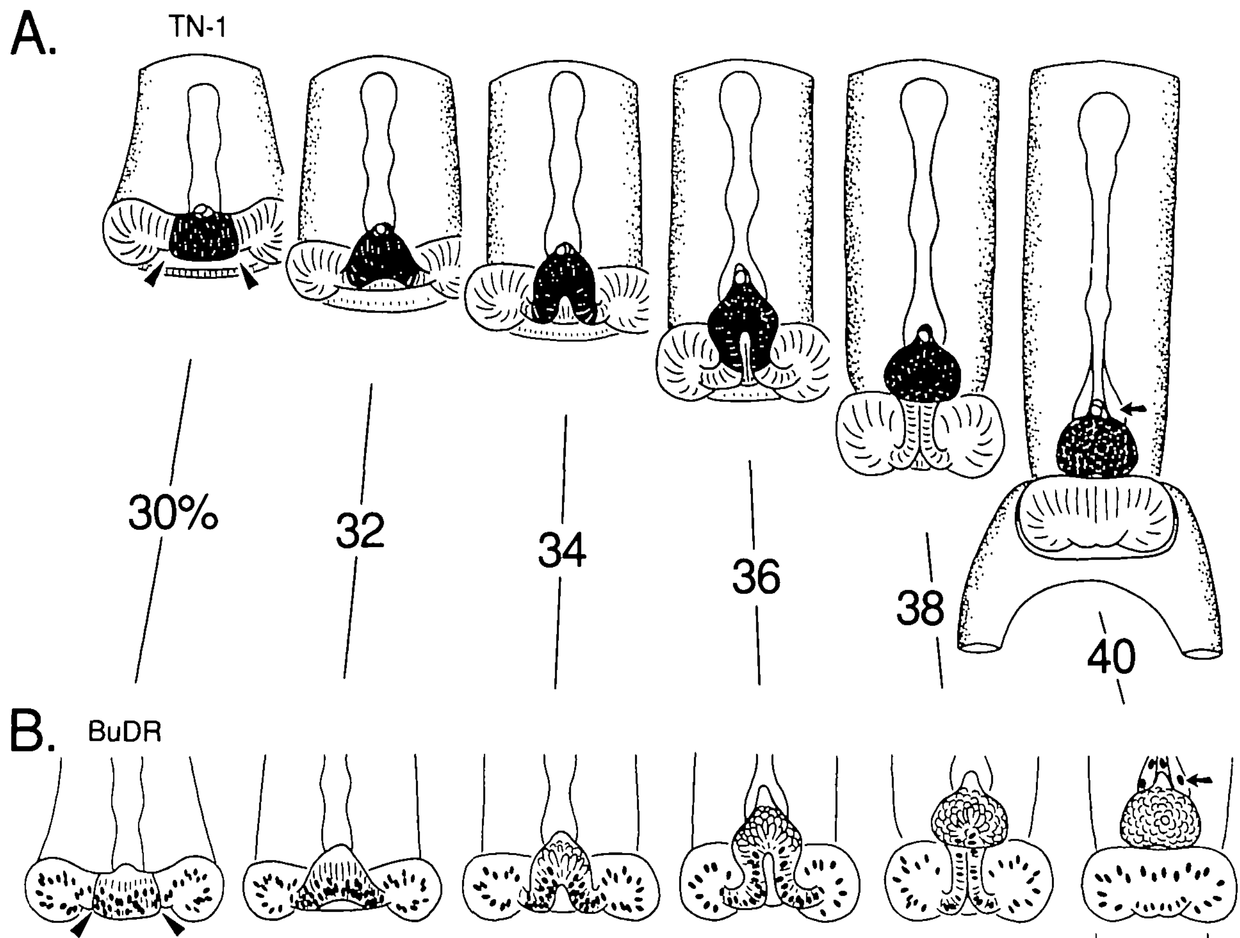


Fig. 2. Generation of the EP cells by placode invagination. (A) Schematic drawings of the embryonic foregut at progressively older stages of development (30–40 %), stained with the monoclonal antibody TN-1 (anterior is up); the immunoreactive cells of the neurogenic placode have been highlighted in black. Arrowheads indicate the boundaries of the placode (also indicated in B and in Fig. 3). The clear tube anterior to the placode on the foregut surface represents the domain of the ENS produced by the anterior neurogenic zones of the foregut. Cells of this domain ultimately establish continuity with the EP cell packet (small arrow). The connection of the foregut with the more posterior midgut anlagen (present throughout this developmental period) has been drawn only in the 40 % stage. (B) Patterns of BrdU labelling (taken from camera-lucida drawings) during the same period of embryogenesis; labelled nuclei are indicated in black. Labelled nuclei occur throughout the foregut epithelium during this time (see Fig. 3H, K), but have been included only in the vicinity of the placode in these drawings. Small arrow indicates a labelled cell derived from the anterior neurogenic zones (same position as in A).

region of the foregut epithelium in a spatially restricted manner and occupied a relatively short period of development (6–10 h). However, the observed patterns of DNA replication did not provide any information concerning the nature of the EP cell precursors, nor of the potential lineal relationships among the different cell types associated with the mature enteric plexus. To better characterize the patterns of division by which the EP neurons are generated, individual cells within the neurogenic placode were filled iontophoretically with a fluorescent lineage tracer at stages between 30 and 40 % of development. Differentiation of the enteric plexus was then allowed to proceed for 24 h in embryo culture, through the phases of placode invagination and EP cell migration (Copenhaver and Taghert, 1989*b*). While individual cells within the placode could not be uniquely identified, clonal analysis of lineage relationships in 82 preparations (as revealed by the distributions of labelled progeny) permitted the following observations.

When individual cells throughout the placode were injected during the initial phases of neurogenesis (30–32 %), a comparatively large number of labelled progeny were subsequently found in the developing enteric plexus (Figs 5A,B; 6D). Typically, between 4 and 8 EP cells were labelled in these experiments, although occasionally only a single fluorescent cell could be detected (Fig. 5C). When injections were performed at later developmental stages (34–36 %), the number of labelled progeny diminished in a stage-dependent manner (Figs 5D,E; 6C). Individual progenitors at these stages gave rise to a maximum of 4 EP cells and more often resulted in only 1 or 2 labelled progeny. The number of labelled progeny also varied

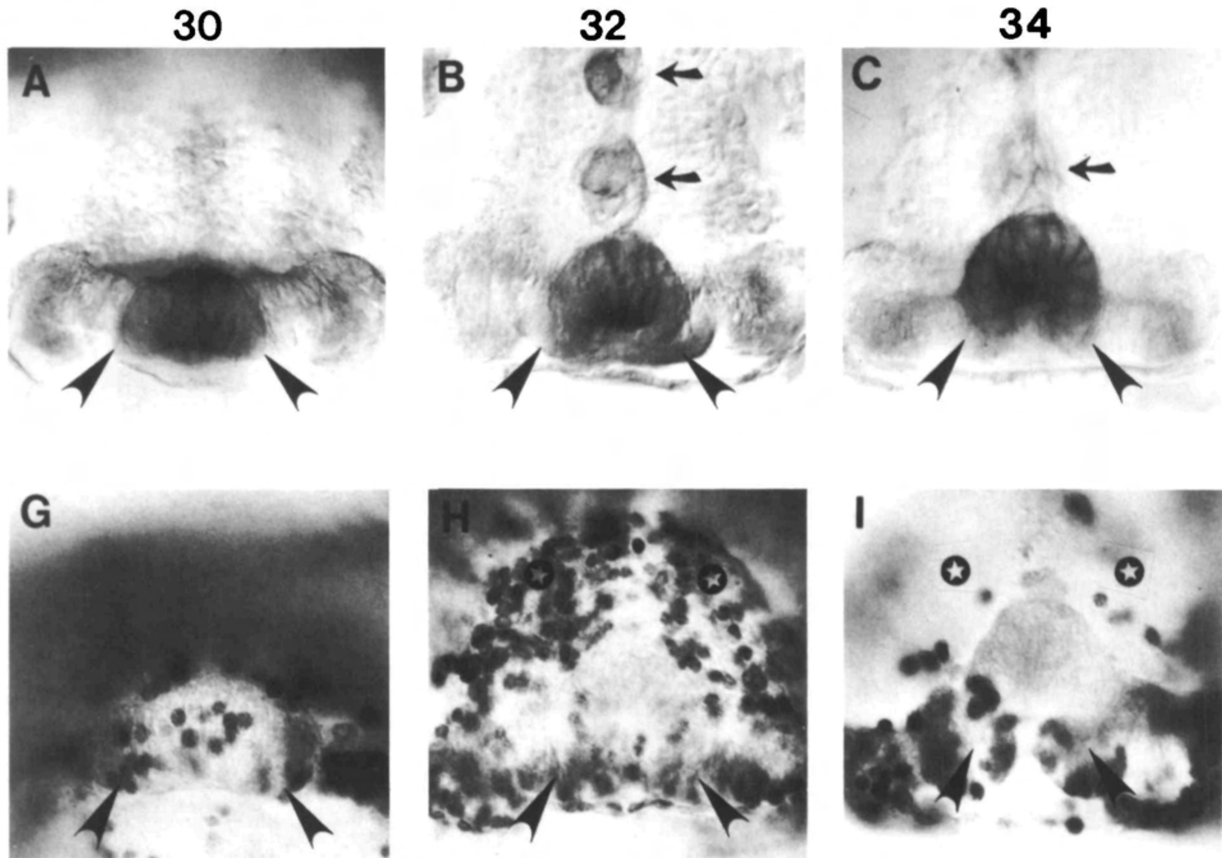
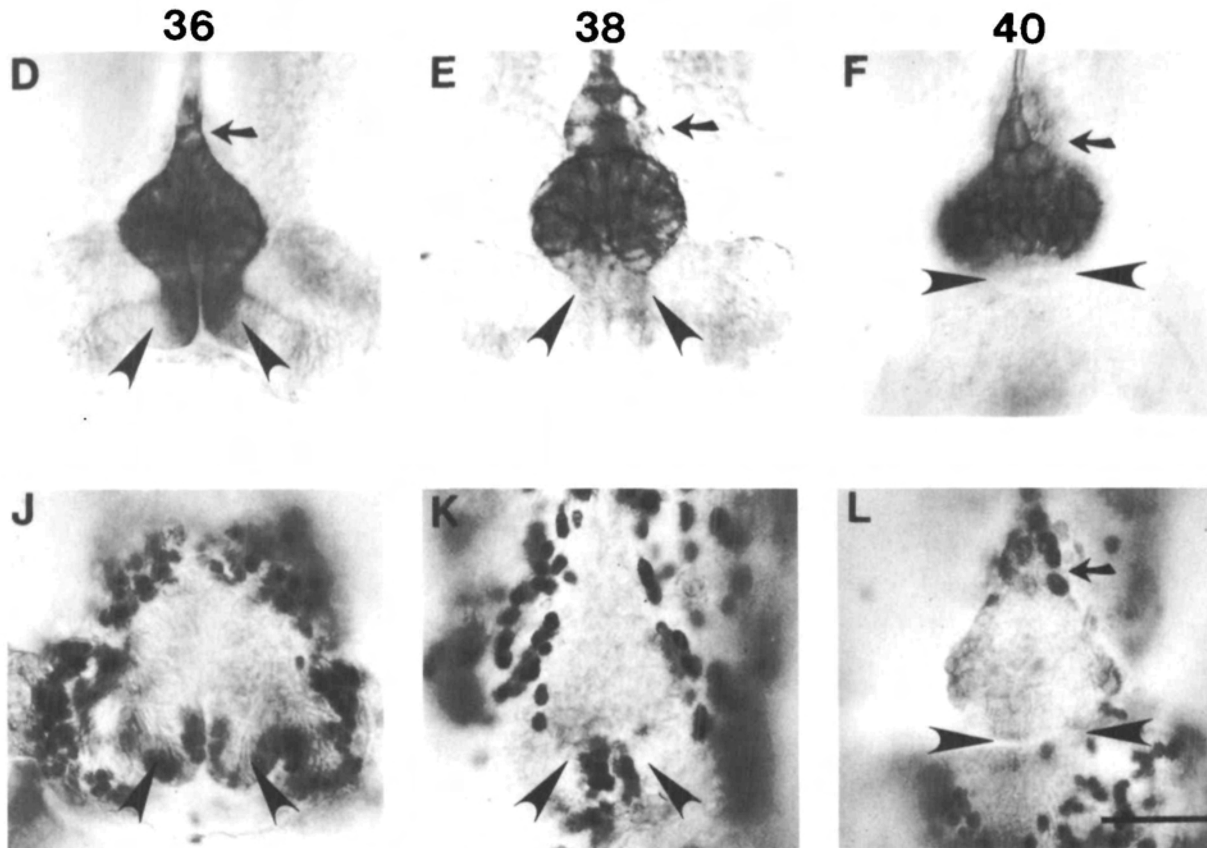


Fig. 3. Photomicrographs of the invaginating EP cell placode at progressive stages of development. TN-1 stained preparations (A–F) and BrdU-labelled preparations (G–L) at comparable ages. Arrowheads indicate the boundaries of the invaginating placode (compare with Fig. 2A). Small arrows indicate cells derived from the anterior neurogenic zones of the foregut (non-EP cells). Stars in H and I indicate gut epithelium that is adjacent to the neurogenic domains of the foregut and which is heavily stained with BrdU labelling throughout this period of development; in I and L, much of this region has been stripped to better delineate the unstained EP cells. Several preparations were also counterstained lightly with TN-1 to enhance the visibility of the EP cells (e.g. I, L). Scale=50 μm.

with respect to the position of the injected progenitor cell in the placode: cells within the epithelial layer of the placode gave rise to the larger numbers of EP cell progeny, while injection of cells that were already rounding out of the epithelium typically resulted in only one labelled neuron. Similarly in all subsequent developmental stages (40 % and older), injection of individual cells within the emerged packet resulted in only a single neuron being labelled (Figs 5G–I, 6B), indicating that no further mitotic divisions had occurred. These results are in agreement with the patterns observed in BrdU-labelled preparations (Fig. 2B), which showed that once removed from the epithelium, the EP cells became post-mitotic.

Invariably, when the progeny of an individual placode cell were incorporated into the developing enteric plexus, they assumed exclusively *neuronal* phenotypes: labelled cells within the EP packet had large, ovoid somata with bipolar processes of varying lengths and continued to express the TN-1 epitope, characteristic of the developing EP neurons (Copenhaver and Taghert, 1989*b*). In contrast, columnar cells at the base of the placode (that remained within the epithelial layer) subsequently gave rise to clones of 20–30 epithelial cells in the foregut wall that were no longer TN-1-positive (Fig. 6E), posterior to the invaginated neuronal population. Notably, while both neurogenic and non-neurogenic cells could be labelled within the lateral margins of the placode, individual cells were never found to contribute progeny to both neuronal and epithelial lineages. Whether the neural and epithelial progenitors are spatially restricted within the lateral margins of the placode or whether the two precursor types are interspersed in this region has not yet been determined. Clonal analyses of the more anterior regions of the developing ENS have revealed labelled progeny with distinctly glial phenotypes (Copenhaver and Taghert, in preparation); the origins and distribution of the glial population that ultimately is incorporated into the enteric plexus will be described in a subsequent report.

Other aspects of EP cell differentiation proceeded normally in these cultured preparations, including cellular migration along defined pathways and the continued elongation of neuronal processes, which could often be followed due to the cytoplasmic diffusion of the injected dye (e.g. Fig. 6B). In addition, the injection protocol used for these experiments did not appear to cause premature cessation of mitosis: injection of



CNS neuroblasts within the brain during similar periods of development consistently yielded a large number of labelled neurons following a 24 h period in culture (Fig. 6F). In a small minority of preparations (<5 %), no labelled cells could be observed following 24 h of development, presumably due to damage or death of the injected cell. At present, we have no evidence for the regulation of EP cell number by systematic or widespread cell death.

These experiments indicate that while many of the mitotically active placode cells contributed progeny to the EP cell packet during the process of invagination, those that did so underwent a limited number of divisions before being extruded onto the foregut surface. Numerical analysis of these preparations showed that a steadily decreasing number of labelled progeny were produced from individual epithelial cells injected during the course of placode invagination (Fig. 7). By 38 %, the generation of the EP cell population was essentially complete, with no substantial modifications in neuronal numbers occurring throughout the subsequent phases of migration and differentiation.

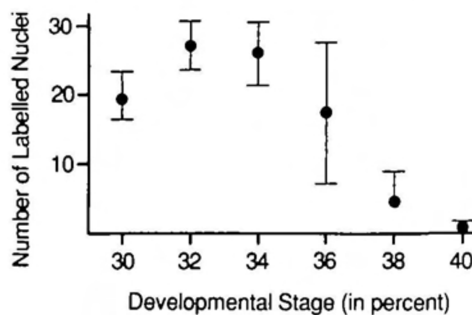


Fig. 4. Numerical analysis of BrdU-labelled nuclei in the invaginating EP cell placode. Each point represents the mean (+S.D.) of cell counts taken from at least 10 preparations per timepoint. Only cells that were within the boundaries of the placode (as defined by morphological criteria or by TN-1 counterstaining) were included in this analysis. Preparations stained after 40 % showed no additional EP cell labelling and are not illustrated in the graph.

Discussion

Neurogenic programs in the insect nervous system

The sequence of events giving rise to the EP cells represents a neurogenic process that has not previously been described in the development of the insect nervous system. In the embryonic CNS (Fig. 8), individual neuroblasts become segregated from the neurectoderm while still mitotic and undergo a stereotyped sequence of asymmetric divisions; these give rise to ganglion mother cells that in turn produce pairs of neurons of predictable phenotypes (Bate, 1976; Goodman and Spitzer, 1979; Taghert and Goodman, 1984). A small number of neurons are generated by the mid-line precursor cells (Bate and Grunewald, 1981; Goodman *et al.* 1981), which also emerge from the epithelium while mitotically active but which only divide once. Newborn neurons then typically proceed with their

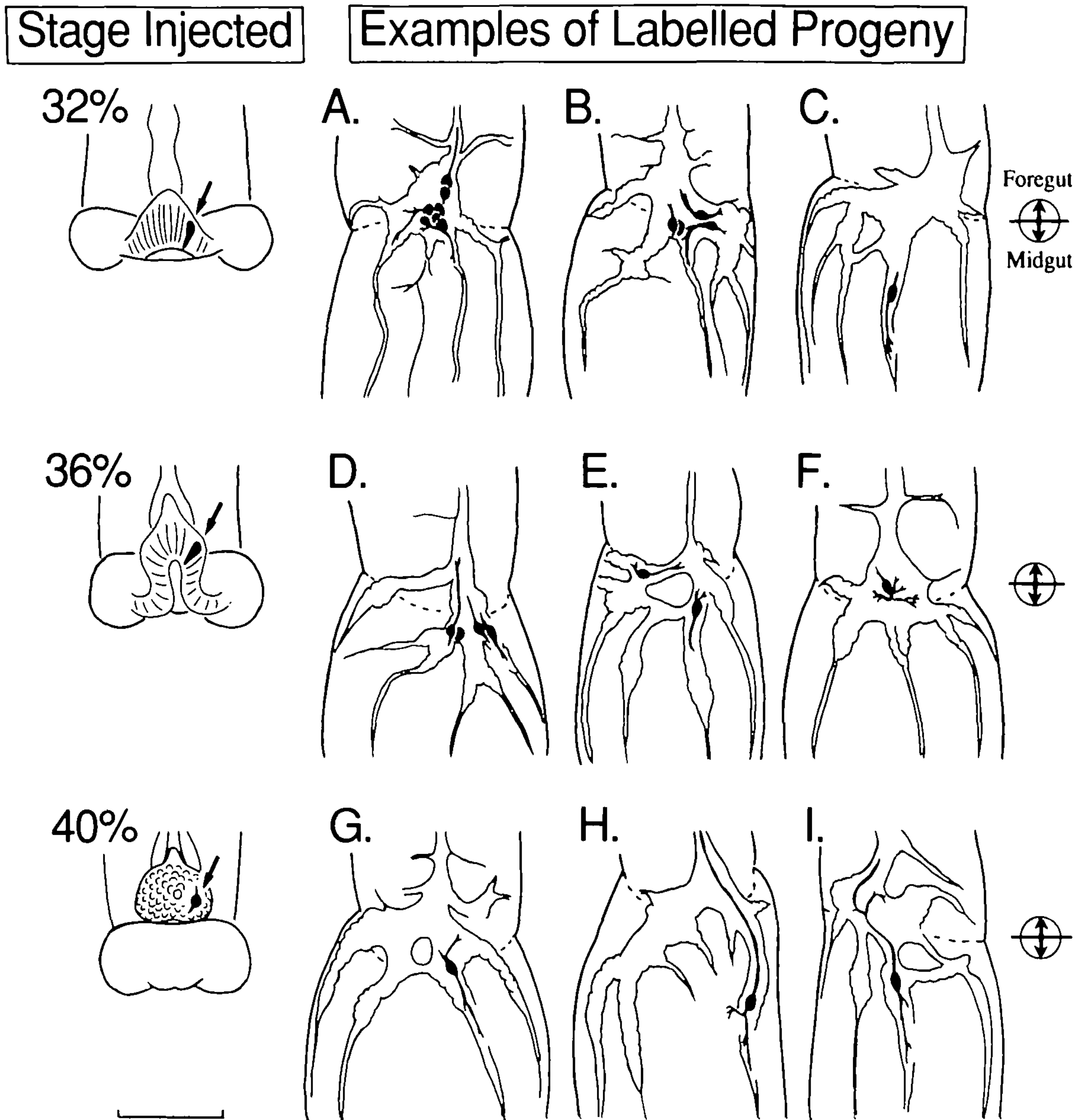


Fig. 5. Patterns of labelled progeny in the enteric plexus following fluorescent dye injections of individual placode cells. The stage at which single placode cells were injected with LRD is indicated on the left (in percent of development). Injected cells are illustrated schematically in black (arrows). Representative examples of labelled progeny after 24 h in culture (drawn by camera lucida) are shown to the right of each injection stage: A, B, and C were injected between 30 and 32 %; D, E, and F were injected between 33 and 36 %; and G, H, and I were injected between 37 and 40 %. The examples shown were chosen to illustrate the range of progeny numbers following single cell injections at the different developmental stages (see Fig. 6). The irregular boundaries of the developing enteric plexus in these preparations (outlined in black on the gut surface) are due to minor perturbations in the overall structure of the foregut–midgut boundary that arise in culture. Scale=200 μm.

differentiation within three or four percent of development and with little or no displacement from their initial location. Similarly in the PNS, embryonic sensory neurons arise from stereotyped precursors (Lawrence, 1966; Technau and Campos-Ortega, 1986; Hartenstein, 1988), each of which produces one or more neurons which commence differentiation soon after birth (Ghysen *et al.* 1986). A modification of this sequence has been seen in neurons born post-embryonically in the insect CNS: these arise from identifiable neuroblasts but do not complete their differentiation until the time of metamorphosis (Booker and Truman, 1987).

In contrast, the EP cells of the ENS are generated as a coherent group from a proliferative epithelium (Fig. 8), becoming post-mitotic in the course of their emergence from the columnar layer. Rather than being generated *via* a sequence of asymmetric divisions from neuroblast-like precursors, the EP cells are produced as small clones from an ensemble of columnar epithelial cells that undergo a limited number of divisions, im-

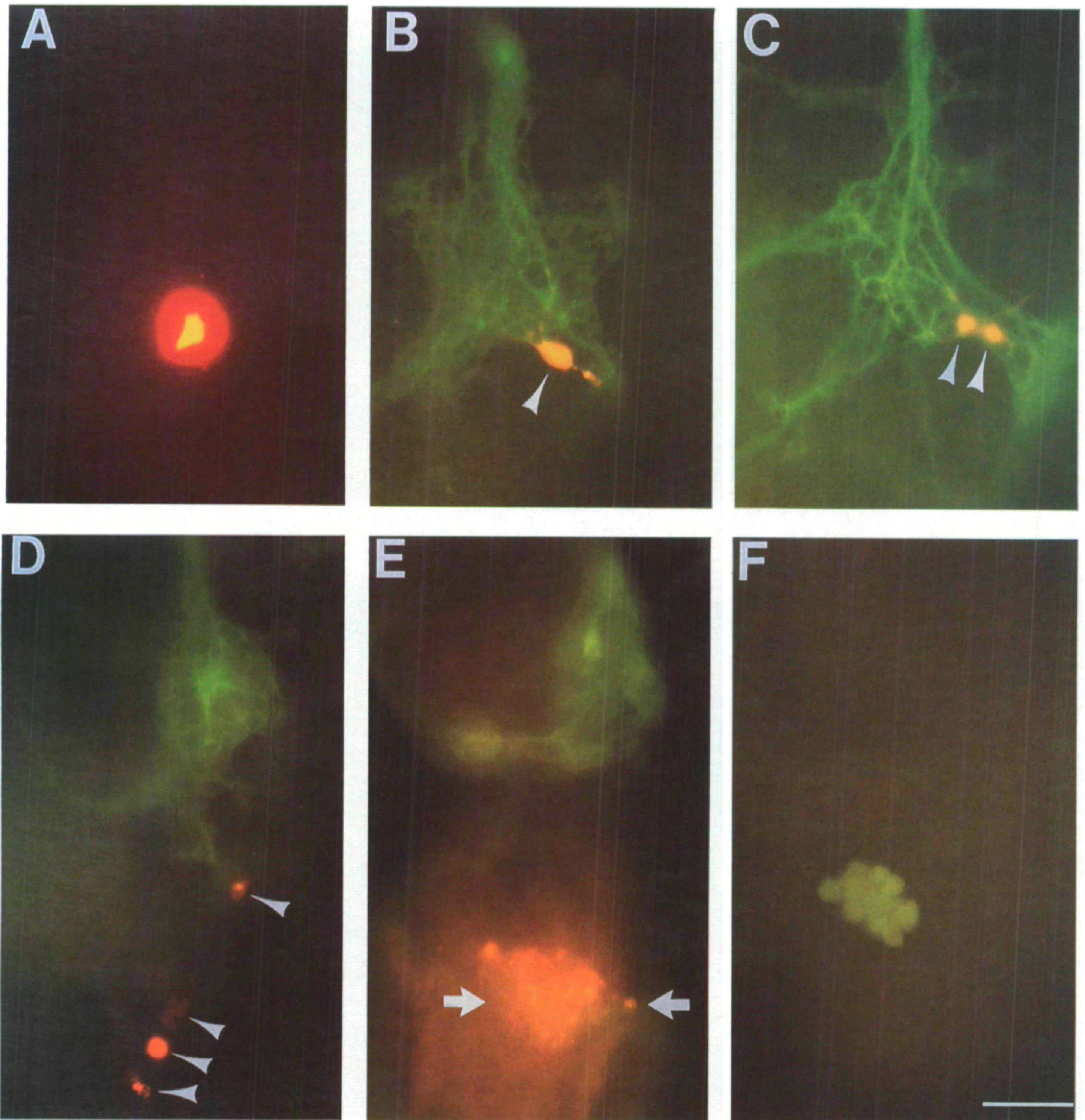


Fig. 6. Photomicrographs of labelled progeny after 24 h in culture, following fluorescent dye injections of single precursor cells. Preparations shown in panels A–E were injected with lysinated tetramethylrhodamine dextran (LRD); F was injected with lysinated fluorescein dextran (LFD). B–E were counterstained immunohistochemically with the monoclonal antibody TN-1 (followed with a fluorescein-conjugated secondary antibody) to reveal the extent of the developing enteric plexus. Panel A shows an example of a single placode cell injected at 32 % of development and fixed immediately; all other embryos were cultured for 24 h following the initial injection of the lineage tracer. Labelled progeny are indicated by the white arrowheads. (B) A single EP cell labelled in the enteric plexus after a placode cell was injected at 38 % of development. The posterior process of this bipolar cell can be seen projecting along the migratory pathway of the developing enteric plexus. (C) Two labelled EP neurons after injection of a placode cell at 36 %. (D) Four labelled EP neurons after injection of a placode cell at 32 % (not all of the cells are in the same focal plane). In this preparation, the cells had undergone extensive migration along the gut surface prior to fixation. (E) Example of the progeny of an epithelial cell filled at 38 % within the lateral folds of the invaginating placode. The cell produced 25–30 cells that became incorporated into the epithelial wall of the developing gut (the fluorescein-counterstained neurons of the enteric plexus can be seen anterior to the labelled gut cells). (F) Labelled progeny of a brain neuroblast injected at 40 % of development. Scale=50 μm.

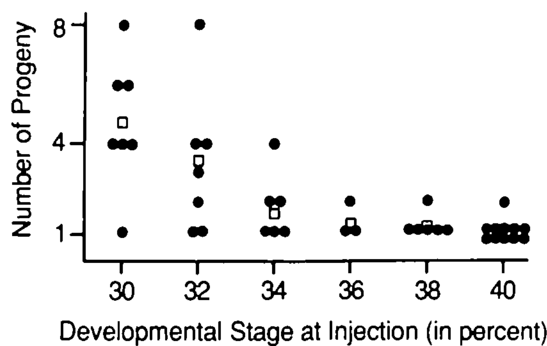


Fig. 7. Numerical distribution of the progeny of dye-injected placode cells after 24 h in culture. The stage at which the initial injection was performed is indicated on the horizontal axis (in percent of development). Each filled circle represents the result from a single preparation. Only animals in which a single placode cell was unambiguously filled (confirmed visually; see Fig. 6A) were included in this analysis. The open squares represent the mean number of labelled progeny for each developmental stage.

mediately followed by their delamination from the epithelial layer. The simplest interpretation of our data would be that an initial population of ~45 progenitor cells arises within the placode, each of which undergoes 3 symmetrical divisions to produce 8 of the 360 EP cells found in the mature plexus. This interpretation is supported by our observations that progressively fewer EP cells were produced by individual placode cells as development proceed (Figs 5, 6), and that the clone sizes from single neurogenic cells were clustered around 1, 2, 4, and 8 (Fig. 7). However, more complex variations in the size of the 'founder' population within the placode and the numbers of their progeny are equally possible. At present, we do not have an unambiguous marker to distinguish neuronal and epithelial precursors within the placode, prior to the differentiation of the cells they produce.

Once generated from the placode, the EP cells acquire neuronal phenotypes exclusively, while the glial cells that subsequently ensheath them arise from a distinct proliferative zone (Copenhaver and Taghert, in preparation). However, as shown previously (Copenhaver and Taghert, 1989*b*), the EP cells delay the expression of their mature phenotypes for up to 35 % of subsequent development (Fig. 8). During this time, they become dispersed by a phase of extensive migration into divergent environments of the enteric plexus (Copenhaver and Taghert, 1989*a*,*b*), and many of the biochemical and morphological characteristics that distinguish the different EP cell types (including the FMRFamide-related phenotype, Fig. 1) are ultimately expressed in a position-specific manner. Together, these observations suggest that neurogenesis in this system involves the recruitment of placode cells *via* a binary decision between ectodermal fates (Harris and McKeown, 1986), a choice that affects both the duration of their mitotically active phase and the phenotypes that their progeny will ultimately express.

In several regards, the developmental events giving

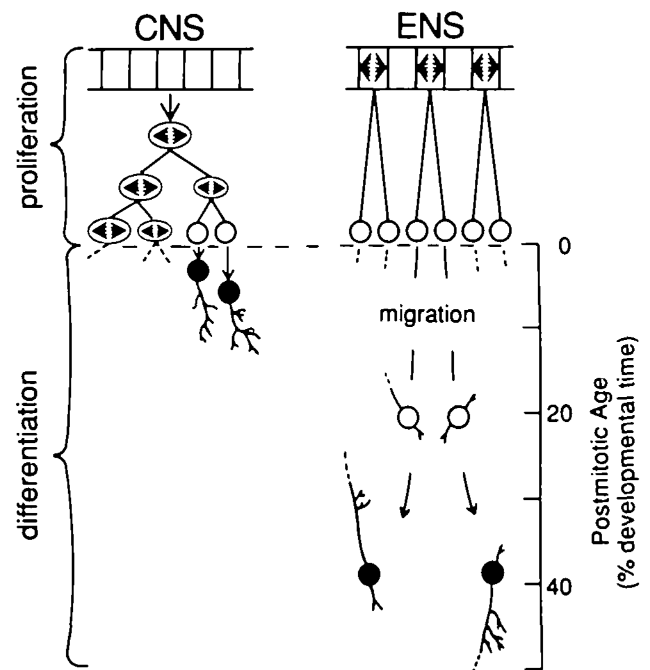


Fig. 8. Comparative models for neurogenesis in different domains of the embryonic insect nervous system. In the CNS, the *proliferation* of neurons commences with the segregation of precursor cells from the neurectoderm, which are mitotically active and which assume unique identities that correspond to their subsequent divisional patterns and progeny types. The emerged neuroblasts (large ovals) typically undergo a stereotyped sequence of asymmetric divisions; these give rise to as many as 50 ganglion mother cells (small ovals), each of which divides once more and produce pairs of neurons of predictable phenotypes (filled circles). The *differentiation* of central neurons typically proceeds soon after their terminal mitosis and with little or no migration. In the ENS, by contrast, the *proliferation* of the EP cells occurs within an epithelial placode, and the neurons (open circles) become post-mitotic as soon as they emerge from the columnar cell layer. Their *differentiation* is then delayed for up to 35 % of subsequent embryogenesis (filled circles), until after their dispersal *via* an extended phase of migration into divergent regions of the enteric plexus.

rise to the insect enteric plexus resemble patterns of neurogenesis previously described in a variety of vertebrate embryos. For example, the majority of neurons in chick cranial sensory ganglia are derived from discrete epithelial placodes (Hamburger, 1961; D'Amico-Martel, 1982). These placodal cells undergo a mesenchymal transformation as they leave the epithelium, migrate from their site of origin, and then coalesce into peripheral ganglia before completing their differentiation (D'Amico-Martel and Noden, 1983; Le Douarin and Smith, 1988). Another example of this developmental sequence has recently been described in the mouse, where neurons derived from the olfactory placode also become post-mitotic upon their delamination and then migrate into the developing forebrain and hypothalamus (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al.* 1989). As with the EP cells of *Manduca*, at

least some of these cells also become peptidergic, producing substances related to luteinizing hormone-releasing hormone; although in this case, the peptidergic phenotype is expressed even before the onset of migration. Thus similar types of cellular processes may regulate the recruitment of epithelial cells towards a neuronal phenotype during ENS formation in *Manduca* as occurs during the epithelial-to-neuronal transformation in these more complex systems.

While morphogenesis from an epithelial placode has not previously been described during the embryonic formation of the insect nervous system, it is a common feature in the differentiation of many other embryonic tissues and organs in invertebrates (Fristrom, 1988). Most secretory glands in insects (including mandibular, salivary, and silk glands), portions of the neuroendocrine system, and cells forming the respiratory tracheal tree originate from invaginated placodes of epithelium (Anderson, 1972; Campos-Ortega and Hartenstein, 1985), as do the imaginal disc cells that subsequently differentiate into adult structures at metamorphosis (Poulson, 1965; Fristrom and Rickoll, 1982; Nardi, 1981). Neurogenic placodes have also been described in the mollusc *Aplysia*, in which each of the CNS ganglia accumulates cells from an adjacent region of proliferative epithelium during both embryonic and post-embryonic development (McAllister *et al.* 1983; Jacob, 1984). Thus as with cell migration (McAllister *et al.* 1983), this mode of neuronal proliferation may prove to be a more prevalent feature of invertebrate development than previously appreciated.

Mitotic patterns and cell lineage in the neurogenic placode

The combined use of BrdU labelling and intracellular lineage tracing techniques has given us insight into the patterns of mitosis by which the enteric plexus neurons are generated: cells within a discrete region of epithelium undergo a limited number of divisions prior to their extrusion from the epithelial layer. An important issue is the validity of the BrdU protocol for the construction of mitotic maps, as this technique will also label cells that are undergoing DNA replication but not preparing to divide (an increase in ploidy). However, if the EP cells became polyploid in the course of their differentiation, we would expect continued or enhanced labelling in the emerged cell population, rather than the complete loss of staining that was observed. Using sectioned material (not shown), we also observed a distribution of mitotic profiles that was consistent with the patterns of DNA replication described in this paper. Moreover, in contrast to most other ectodermally derived cell types, insect neurons classically have not been found to become polyploid in the course of their differentiation (Anderson, 1972) – a finding that has been augmented by other studies of DNA replication in the insect nervous system (Truman and Bate, 1988; Bodmer *et al.* 1989). Lastly, our investigation of cell lineage relationships within the placode confirmed the overall patterns of replication revealed by the BrdU technique. Numerical analyses have shown that the vast majority of EP cells in the mature plexus can be accounted for at this time (Copenhaver and Taghert, 1989*b*), indicating that the combined processes of placode invagination and epithelial delamination represents the primary neurogenic sequence by which this class of enteric neurons is generated.

Clonal analysis of individual placode cells allowed us to determine the patterns of division that gave rise to the EP cell population: a large proportion of mitotically active epithelial cells underwent a limited number of divisions and contributed progeny to the enteric plexus, in contrast to the neuroblast-derived lineages found in the insect CNS. However, several issues concerning specific mitotic relationships within this neurogenic placode remain unexplored. For example, placode cells were found to contribute progeny either to the enteric plexus or to the gut epithelium but not both. The degree to which mitotic ancestry governs the commitment to these alternative developmental pathways remains obscure. Also uncertain is whether all of the neurogenic cells give rise to equal numbers of EP cells, or whether other factors regulate progeny number – such as the interval between a placode cell's recruitment into the neurogenic pathway and the completion of placode invagination (when EP cell generation is complete). Conversely, the process of placode formation and invagination may ultimately regulate the number of EP cells that are produced. As noted above, the columnar cells that become neurogenic contribute a relatively small number of neurons to the EP cell packet (Fig. 7), while epithelial cells adjacent to the invaginated placode continue to proliferate for many more rounds of division (Fig. 6E). Possibly, the placodal environment supplies local mechanical or chemical constraints on the mitotic activity of the neurogenic cells, thereby limiting the number of divisions that they undergo.

Previous work has shown that as a population, the EP cells emerge from the epithelium in an undifferentiated form but subsequently exhibit a predictable array of morphological and biochemical phenotypes (Copenhaver and Taghert, 1989*a*,*b*). Although the expression of many of these characteristics is delayed until after EP cell migration is complete (Copenhaver and Taghert, 1989*b*), the developmental potential of individual neurons may nevertheless be constrained by lineage-derived factors. For example, individual placode cells might give rise to a particular class of EP cell or to an orderly progression of cellular subtypes. The combined application of our lineage-tracing protocol with an extended analysis of neuronal differentiation will be required to address this issue.

Placode formation, cell proliferation and epithelial morphogenesis

Placode formation is a consistent feature during the morphogenesis of both invertebrates and vertebrate embryos (Wessells *et al.* 1971; Ettensohn, 1985), but the mechanisms that regulate the diversity of epithelial rearrangements originating from these structures are poorly understood (for review, see Fristrom, 1988). For example, the initial specification of placode size and

position must clearly depend on antecedent events in epithelial development. In this regard, it is noteworthy that the EP placode of *Manduca* is generated at a site that, in part, is already neuralized. Prior to the formation of the placode, three small neurogenic zones form within the foregut epithelium and eventually give rise to the anterior regions of the ENS (Copenhaver and Taghert, in preparation). The most posterior of these zones originates from the same epithelial region that later is encompassed by the EP placode, although the zone has ceased to elaborate cells by the onset of placode formation. The generation of the placode thus represents a second phase of a more complex neurogenic sequence within this ectodermal region, which will be described in a subsequent report.

A second major issue concerning the mechanisms of epithelial transformation is the role of restricted patterns of mitosis during the morphogenetic process. In at least some systems, extensive cellular rearrangements can continue even when cell division is completely blocked, such as in amphibian gastrulation (Cooke, 1978) and imaginal disc morphogenesis in *Drosophila* (Siegel and Fristrom, 1978). Alternatively, differential patterns of mitosis can play a morphogenetic role when the cell divisions are localized to particular domains of an epithelial layer (Fristrom, 1988). Such a localization was observed during ENS formation in *Manduca*: the distribution of synthetically active nuclei became increasingly confined to the outer limits of the placode, where the most pronounced folding of the epithelial sheet occurred during the invagination process (Fig. 3). Recently, we have also found that placode invagination will proceed to completion even when the stomodeal rudiment is cultured in isolation from the rest of the embryo (unpublished observations). Thus the generation and elaboration of the EP placode is autonomous to a relatively small region of the foregut, a region that can now be made accessible to experimental manipulation *in vitro*.

Taken as a whole, the events that give rise to the enteric plexus suggest a coherent program of neurogenesis: generation of an undifferentiated cell population from a proliferative zone of epithelium, migratory dispersal along divergent pathways, and the delayed expression of neuronal phenotypes in a position-specific manner. As such, this neurogenic sequence would represent one of several developmental programs available for the construction of an embryonic nervous system, distinct from the more stringent programs involving stereotyped lineages that direct CNS development in insects and other invertebrates. Perhaps these alternative strategies for neurogenesis permit varying degrees of plasticity in different domains of the nervous system, corresponding to the physiological requirements of different organ systems. For example, in contrast to many cells of the insect CNS, the EP cells are not uniquely identifiable but comprise distinct subtypes that differentiate in particular domains of the enteric plexus following migration. While the functional roles of the insect ENS are poorly characterized, it will be useful to examine the physiological significance of EP cell distributions with respect to the neurogenic strategy that governs their development.

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