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Gangliogenesis in leech: morphogenetic processes leading to segmentation in the central nervous system

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Abstract Using intracellular lineage tracers to study the main neurogenic lineage (N lineage) of the glossiphoniid leech embryo, we have characterized events leading from continuous columns of segmental founder cells (nf and ns primary blast cells) to discrete, segmentally iterated ganglia. The separation between prospective ganglia was first evident as a fissure between the posterior boundary of nf- and the anterior boundary of ns-derived progeny. We also identified the sublineages of nf-derived cells that contribute parallel stripes of cells to each segment. These stripes of cells project ventrolaterally from the dorsolateral margin of each nascent ganglion to the ventral body wall. The position and orientation of the stripes suggests that they play a role in forming the posterior segmental nerve; they are not coincident with the ganglionic boundary, and they form well after the separation of ganglionic primordia. Previous work has shown that cells in the anterior stripe express the leech *engrailed*-class gene. Thus, in contrast to the role of cells expressing *engrailed* in *Drosophila*, the stripes of N-derived cells expressing an *engrailed*-class gene in leech do not seem to play a direct role in segmentation or segment polarity.

Key words Gangliogenesis · Leech · Annelid · *Engrailed* · Nerve formation

Introduction

Forming the nervous system requires not only the genesis and differentiation of specific neurons and glia, but also the morphogenetic processes by which these cells become organized spatially. Glossiphoniid leeches, such as *Theromyzon rude*, are well-suited for studying the role of cell lineage and cell-cell interactions in development because their embryos are large (~800 μm for *T. rude*) and hardy; in addition, their embryos undergo stereotyped cleavages that give rise to identifiable cells, accessible for experimental manipulation throughout development. Also, the adult leech central nervous system (CNS) is relatively simple in terms of its organization. The bilaterally symmetric CNS comprises a rostral, unsegmented, supraesophageal ganglion and 32 segmentally iterated ventral neuromeres: 4 fused neuromeres make up the anterior subesophageal ganglion; 7 fused neuromeres form a caudal ganglion associated with the posterior sucker; the remaining 21 neuromeres occur as distinct ganglia in the midbody of the animal, separated from adjacent ganglia by interganglionic connective nerves (Stent et al. 1992). Each segmental ganglion contains approximately 400 neurons (Macagno 1980), most of which are bilaterally paired. Many neurons in the adult leech have been assigned individual identities on the basis of morphological, physiological, and/or biochemical criteria (Muller et al. 1981).

Previous work has established that the segmented nervous system, nephridia, epidermis, and musculature of the leech arise from rostrocaudally arrayed columns of segmental founder cells (blast cell bandlets), via intermediate structures called the germinal bands and germinal plate (see Fig. 1). The blast cell bandlets arise from five bilaterally paired stem cells (M, N, O/P, O/P and Q teloblasts) and are initially continuous (i.e., unsegmented). Within the germinal bands, they occupy discrete mesodermal and ectodermal layers, with the mesodermal (m) bandlet lying between the prospective endoderm (the yolk-filled macromeres) and the four ectodermal bandlets (n, o, p and q; Fig. 1; Whitman 1887; reviewed by Stent et al. 1992). As in other triploblastic animals, the leech nerve cord is primarily of ectodermal origin, yet lies within the mesodermally-derived coelom. Thus, the nerve cord is separated from the other major

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ectodermal derivative, the epidermis, by layers of longitudinal, circular and oblique muscle fibers. Most (260–320) of the neurons in an adult ganglion are descendants of the bilateral N teloblasts (Kramer and Weisblat 1985).

In this study, we investigated the processes by which neural precursors become re-organized from continuous, superficial columns of cells (the ectodermal bandlets) into discrete, segmentally iterated ganglia. For this purpose, we followed the time course of gangliogenesis by differentially labeling the N lineage with fluorescent lineage tracers. By microinjecting individual primary and secondary blast cells, we also determined the contribution of specific blast cell progeny at selected times during development. We were particularly interested in relating events of gangliogenesis to the formation of two segmentally iterated transverse stripes of N-derived cells. As described previously (Wedeen and Weisblat 1991; Lans et al. 1993; Ramirez et al. 1995), these stripes of cells transiently express the leech *engrailed*-class gene during gangliogenesis. Based on the apparent homology between the stripes of *engrailed*-class gene expression in leech and arthropods, we previously postulated that the leech *engrailed*-class gene played a role in segmenting the CNS (Ramirez et al. 1995). We present evidence here that these cells may instead play a role in forming one of the segmental nerves by which ganglia innervate the body wall.

Materials and methods

Embryos

Theromyzon rude embryos were obtained from specimens collected in the lakes of Golden Gate Park, San Francisco, and were cultured as previously described (Torrence and Stuart 1986), except that they were maintained at 23°C. Primary blast cells and their progeny were designated according to the system of Zackson (1984), as extended by Bissen and Weisblat (1989).

Lineage tracer injections

Fluorescent lineage tracer [either fluorescein-dextran amine (FDA; Molecular Probes) or tetramethylrhodamine-dextran amine (RDA; Molecular Probes)] was injected into the N teloblast after its birth as previously described (Weisblat et. al. 1980b). To follow the development of individual blast cell clones, primary (nf, ns, at 7–12 h clonal age; see next paragraph) or secondary (nf.a, nf.p, ns.a, ns.p, at clonal age ~20 h) blast cells were injected with a second lineage tracer. Both teloblast and blast cell injections were performed under a dissecting microscope. Successful teloblast injections were those in which the embryo survived and the injected cell produced a well-labeled, normal complement of progeny, as judged by a continuous column of primary blast cells in the early embryo and the normal complement of segmentally iterated definitive progeny in the older embryo as described previously (Weisblat et al. 1984; Weisblat and Shankland 1985; Kramer and Weisblat 1985). Successful blast cell injections were those in which the size, shape and distribution of labeled cells in the segment containing the doubly labeled clone were consistent with those of the segments anterior and posterior to it. Unsuccessful injections were those in which the embryo or injected cell died, or gave rise to broken columns of primary blast cells or irregular distributions of blast cell progeny. By these criteria, the rate of successful injections was about 90% for teloblasts and 30% for blast cells. Blast cell injections were carried out without attempting to identify the specific cells being injected, so the estimated success rate is averaged over all types of cells. By this procedure, we obtained roughly ten times as many successful injections of nf.a cells as of nf.p cells and similar ratios were obtained for ns.a versus ns.p injections. This discrepancy probably reflects differences in relative size of the secondary blast cells, and perhaps also differences in their hardiness and/or their positions within the germinal bandlet. Overall, more than 200 *T. rude* embryos were dissected for the observations reported here.

Clonal age determination

The age of cell clones derived from primary or secondary n blast cells in *T. rude* embryos was determined relative to the age of the first labeled clone in the N lineage. The rate of blast cell genesis was determined empirically by injecting the N teloblast with RDA and allowing development to proceed for several hours before fixation. The number of labeled blast cells was then divided by the time interval between teloblast injection and fixation. From this we determined that primary blast cells were produced at a rate of approximately 1.6 cells per hour at 23°C. Thus, assuming that the teloblast cell cycles producing nf and ns blast cells are of equal length in *Theromyzon*, as in *Helobdella* (Bissen and Weisblat 1989), consecutive n blast cells differ in age by about 0.6 h. Using this number, the age of any given primary or secondary n blast cell clone was estimated by counting the clones between it and the leading edge of a labeled bandlet. The error of this method was approximately ± 1 h due to the time span in which embryos were injected and the time it takes a cell to recover from the trauma of injection (Bissen and Weisblat 1989).

Determination of the number of N lineage progeny at specific time points

Primary blast cell clonal progeny were counted for a total of 46 clones at time points ranging from 20–90 h clonal age, using serial optical sections obtained by confocal microscopy. Outlines of cells labeled with lineage tracer were readily discerned by sectioning through focal planes comprising the progeny of a given clone.

Histochemistry

Embryos were fixed in 2% formaldehyde [in 0.2 M 4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2] containing Hoechst 33258 (1 mg/ml final concentration) overnight at 4°C. The vitelline membrane was removed manually, and the germinal plate was dissected from the yolk with fine pins (Fine Science Tools, Cat. No. 10130-05). Germinal plates were mounted in 80% glycerol containing 4% n-propylgallate; for viewing, embryos were placed on a slide, drawn out of the glycerol solution with pins and covered with a cover slip.

Microscopy

A Zeiss Axiophot microscope was used to examine and photograph dissected germinal plates. Slides were taken using Ektachrome 400 film (Kodak) and scanned with a SprintScan 35 Plus (Polaroid) slide scanner. Image enhancement (e.g., adjustment of color levels, merging of images) was performed with Adobe Photoshop (version 4.0). Confocal microscopy was performed with a MRC 600 system (Bio-Rad) attached to a Nikon epifluorescence microscope with a ×40 oil immersion lens. A z-series for each N lineage clone was collected in fluorescein and rhodamine channels at intervals of 1–2.5 μm. For some color images, a z-series was projected onto a single plane using SOM software (Bio-Rad). In

some preparations, relative clonal volumes were estimated from confocal z-series by tracing the contour of the clone for each confocal section, then cutting and weighing the contours for each clone.

Embryo sectioning

To obtain transverse views, germinal plates were prepared from fixed *T. rude* embryos as described above, then dehydrated through a graded series of alcohols (50–95%) and embedded in glycol methacrylate resin (JB-4; Polysciences) according to the manufacturer's instructions. The embedded embryos were then cut into sections roughly 20 μm thick using glass knives and a Sorvall MT2-B ultramicrotome. Sections were placed onto dry, gelatin-coated slides, then expanded by the addition of water drops. The slides were re-dried and coverslips were mounted in either 80% glycerol in 100 mM TRIS, pH 9.0, or the glycol methacrylate embedding medium. Sections were viewed and photographed as described above.

Results

Overview

An overview of the events leading from blast cell formation to discrete ganglia was obtained by injecting N teloblasts with lineage tracer and examining the labeled embryos at various times thereafter. For this purpose approximately 50 dissected germinal plates were examined, from embryos in which both N teloblasts had been injected with lineage tracer (see Materials and methods). In such preparations, the great similarity in midbody segments and the strict anteroposterior progression of development allowed us to infer the temporal sequence of events in a typical midbody segment by examining consecutive segments within the germinal plate. A summary of these results is outlined in Fig. 1, and representative preparations of *T. rude* embryos fixed at relevant embryonic stages are shown in Fig. 2. Briefly, alternating nf and ns primary blast cells formed a column of cells as they emerged from the bilateral pair of N teloblasts (N_L and N_R). At clonal age 18–19 h, primary nf blast cells divided unequally to generate two secondary blast cells, a larger anterior cell (nf.a) and a smaller posterior cell (nf.p). The ns blast cell underwent a more equal mitosis at clonal age ~20 h, generating anterior (ns.a) and posterior (ns.p) cells. These and subsequent cell divisions are oriented such that the n bandlets remain as single columns of cells as the germinal bands coalesce to form the germinal plate (clonal age ~40 h; Fig. 2 K, L; Bissen and Weisblat 1989). Following coalescence, further divisions of blast cell progeny led to the formation of segmentally iterated bulges within the n bandlets (Fig. 2 I, J). Between clonal ages 45–50 h, a transverse cleft appeared near the lateral edge of each bulge. These clefts elongated medially and met at the ventral midline, forming a fissure that subdivided the N-derived cells into prospective ganglia. Later (clonal age ~60 h), two laterally directed stripes of cells emerged from the posterior region of each nf clone (Fig. 2E–H). As described previously (Ramirez 1995), and in homology with the leech *Helobdella triserialis* (Wedeen and Weisblat 1991; Tsubokawa and We-

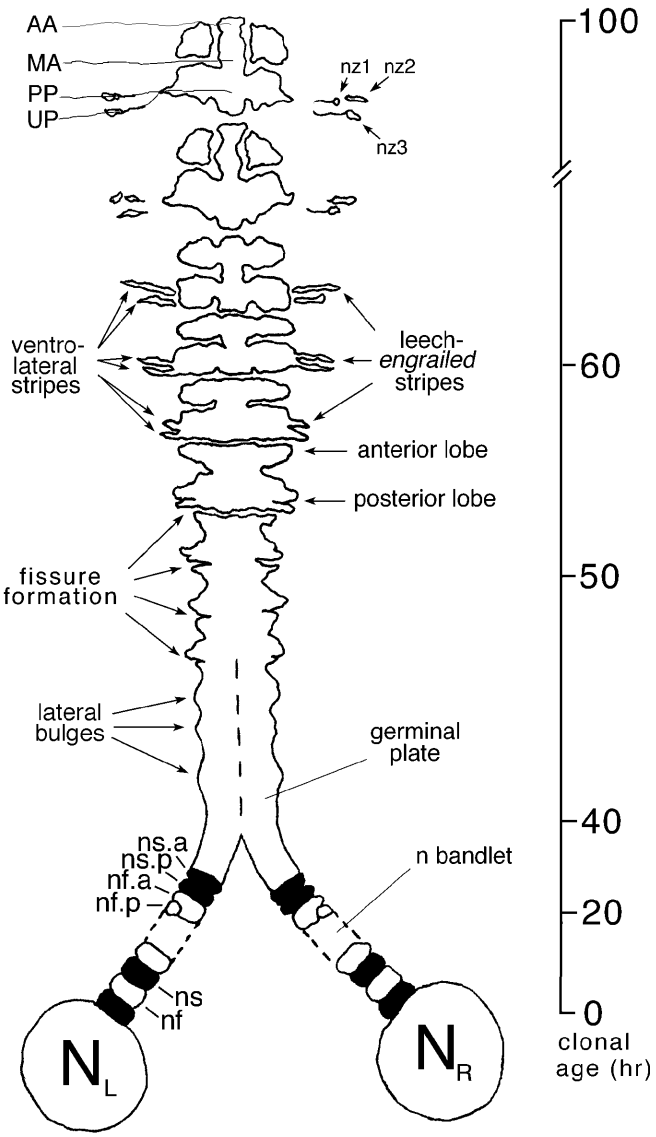
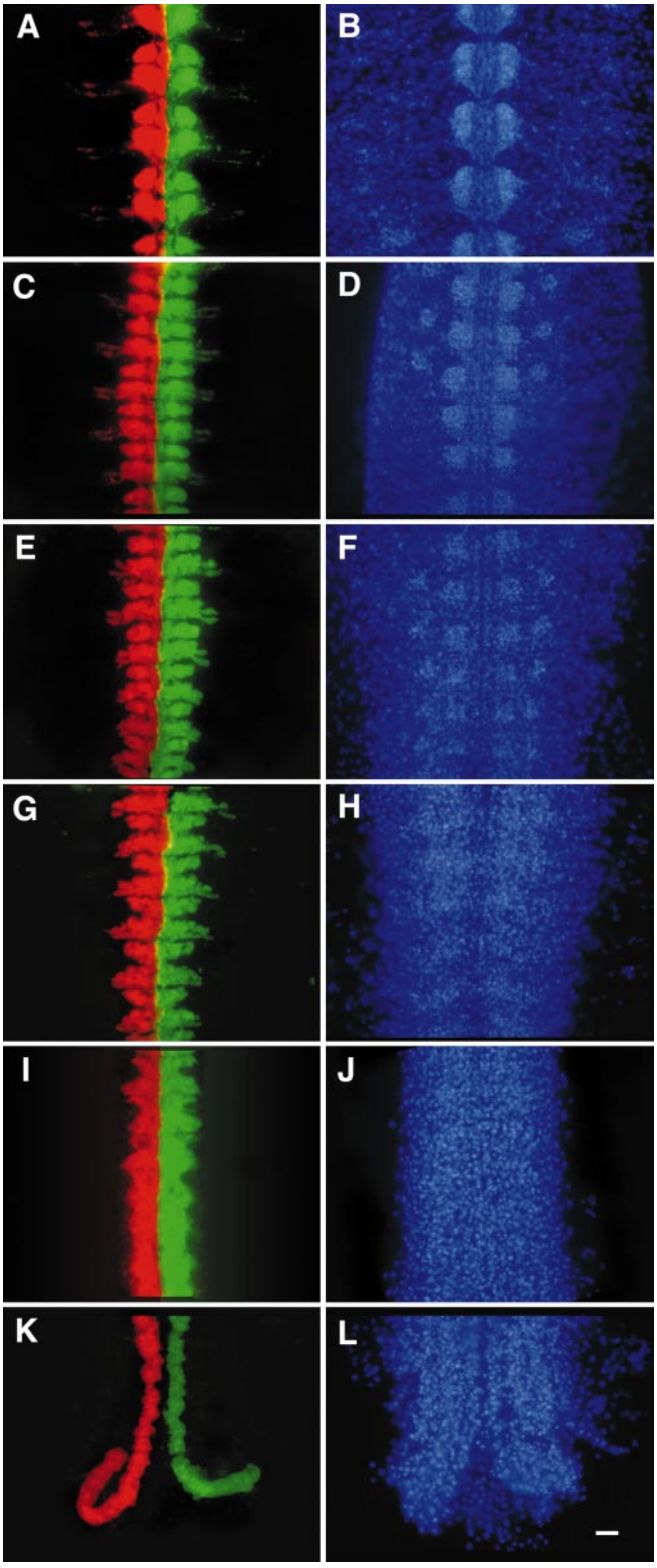


Fig. 1 Composite drawing showing the temporal progression of morphogenetic events in the N lineage during formation of midbody ganglia in the CNS of *Theromyzon* rude. (Contributions from the four other lineages are not shown.) Bilaterally paired N teloblasts (N_L and N_R) give rise to coherent columns of cells (*n bandlets*). Each bandlet comprises two alternating classes of primary blast cells (*ns* and *nf*), whose distinct fates are first indicated by the differences in size of their respective progeny (*ns.a* and *ns.p*, *nf.a* and *nf.p*). Upon entering the germinal plate, contralateral n blast cell clones align along the ventral midline (*dashed line*) and subsequently give rise to the bulk of the segmental ganglia of the ventral nerve cord, along with some segmentally iterated peripheral neurons (*nz1*, *nz2* and *nz3*) and a few epidermal cells (not shown). Morphogenetic processes associated with gangliogenesis include: formation of lateral bulges through proliferation of n blast cell clones; formation of transverse fissures that separate ganglionic primordia and divide the lateral bulges into distinct anterior and posterior lobes; outgrowth of two ventrolateral stripes of cells from each posterior lobe (the anterior of which expresses the leech *engrailed*-class gene); and neural differentiation, including outgrowth of segmental nerves, designated *AA* (anterior-anterior), *MA* (medial-anterior), *PP* (posterior-posterior) and *UP* (ultra-posterior). In addition, neurons project longitudinally via bilaterally paired connective nerves (not shown) and a median unpaired nerve tract (Faivre's nerve; not shown). Approximate clonal ages for n blast cells and their derivatives are indicated *at right*. Anterior is *up*. Not drawn to scale

deen, personal communication), cells in the more anterior stripe (~6 cells) transiently express the leech *engrailed*-class gene.

By clonal age ~75 h, most cells in both stripes had disappeared (Fig. 2C, D), leaving only the previously described peripheral neurons nz1, nz2 and nz3 (Braun and Stent 1989a; Lans et al. 1993). Most features of the mature ganglia were observed by clonal age 100 h (Fig. 2A, B), including the projection of axons along the segmental nerves designated AA (anterior-anterior), MA (medial-anterior), PP (posterior-posterior), and UP (ultra-posterior). Cell proliferation in the nf and ns clones is depicted graphically in Fig. 3.



Formation of a linear array of segmental founder cells: clonal ages 0–45 h

Previous analyses of the N lineage in clitellate annelids have shown that two primary n blast cells are required to

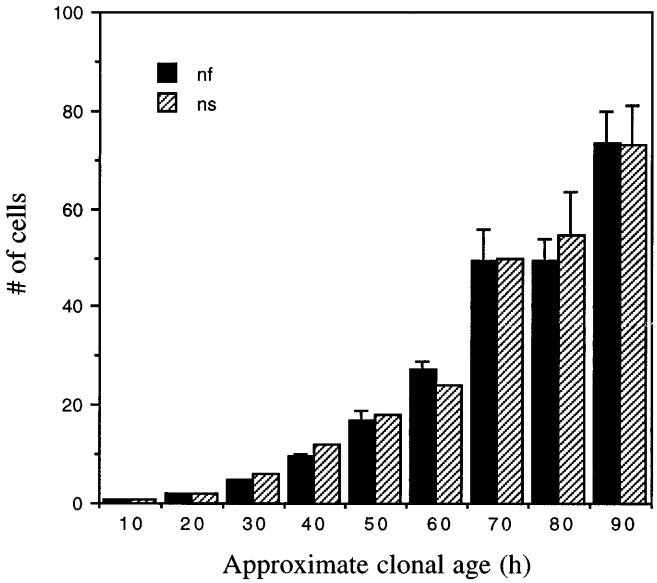


Fig. 3 Proliferation of nf and ns clones during gangliogenesis. The cells in 46 individually labeled primary blast cell clones were counted (see Materials and methods) at approximate clonal ages ranging from 32 to 95 h. The results are grouped within 10 h intervals centered on the times indicated. *Each bar* represents data from 1–8 independent clones; *error bars* indicate the standard deviation for bins with multiple data points. For the first two intervals (5–15 h and 15–25 h), clone sizes (1 cell and 2 cells, respectively for both nf and ns clones) are known by direct observation of the first mitoses and their homology to those in the closely related genus *Helobdella* (Zackson 1984; Bissen and Weisblat 1989)

◀

Fig. 2A–L Key stages during gangliogenesis in *T. rude*. Each panel *on the left* is a double exposure fluorescence photomicrograph showing several segments from the germinal plates of embryos in which the N_L teloblasts were injected with tetramethylrhodamine-dextran amine (RDA; *red*) and the N_R teloblasts with fluorescein-dextran amine (FDA; *green*); the resultant embryos were fixed and dissected at progressively later developmental stages. Panels showing developmentally more advanced segments are *at the top* of the figure, to match the developmental gradient in the germinal plate. In the double exposure photomicrographs (*left panels*), *yellow areas* result from slightly overlapping images caused by imperfect alignment of the two filter sets. The *right panel in each pair* shows nuclei in the same portion of the germinal plate, revealed by counterstaining with Hoechst 33258 (*blue*); *lighter areas* correspond to higher nuclear densities. The range of clonal ages within each panel is as follows: **A, B** 98–102 h; **C, D** 76–84 h; **E, F** 66–74 h; **G, H** 53–59 h; **I, J** 43–51 h; **K, L** 32–40 h (*Scale bar* 20 μm)

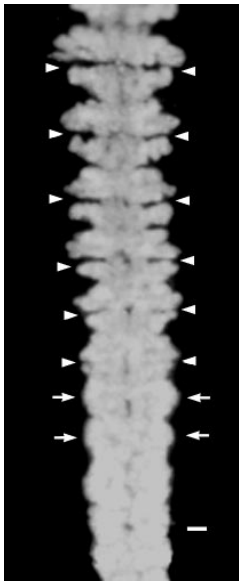


Fig. 4 Fissure formation. Photomicrograph showing roughly ten segments of a germinal plate in which both n bandlets were labeled with RDA (*white*); the ventral midline runs through the center of the long axis (cf. Fig. 2). *Arrows* mark the two most anterior pairs of lateral bulges in which the fissure has not yet formed. *Arrowheads* indicate the position of the fissure on either side of the midline. Blast cell clones in the marked segments range from ~42 h (*bottom*) to ~52 h (*top*). Anterior is *up* (*Scale bar* 20 μm)

make a single segmental complement of N-derived progeny, and that primary n blast cells adopt distinct nf and ns fates in exact alternation at some point prior to their first mitoses [described for *Helobdella triserialis* (Hirudinea) by Weisblat et al. 1980a; Zackson 1984; Bissen and Weisblat 1987, and for *Eisenia foetida* (Oligochaeta) by Storey 1989]. Since there are two n blast cell clones per segment, the length of the prospective segmental ganglion within the bandlet is the length of two primary blast cells (40–50 μm in the fixed specimens of *T. rude* used in our experiments).

As described by Lans et al. (1993), there is a disparity in the age of n and q primary cell clones relative to consegmental m, o and p clones, and this disparity increases as one progresses from anterior to posterior segments. One consequence of this was that anterior n blast cell clones were approximately ~40 h of age upon entering the germinal plate, while posterior clones were only ~25 h when they entered the germinal plate (data not shown). Accordingly, the size of the nf and ns clones at coalescence (Fig. 2K, L) varied from ~2 to ~10 cells between anterior and posterior regions of the germinal plate (Fig. 3).

Fissures appearing between the nf.p and ns.a clones separate ganglionic primordia: clonal ages 45–50 h

Within the germinal plate, the n bandlets eventually lost their linear geometry and formed segmentally iterated bulges by clonal age 45 h (Fig. 2I, J). Presumably, this shape change resulted from oriented cell divisions and/or cell rearrangements as the nf and ns clones increased to ~25–30 cells each (Fig. 3). Cell growth may have also contributed to this process, since the combined volume of the nf and ns clones at this clonal age (~50 h) was at least 50% greater than the combined volumes of primary nf and ns blast cells.

The overt separation of the n bandlets into discrete ganglionic primordia began between 45–50 h clonal age with the formation of bilateral transverse clefts, just posterior to the most lateral extent of the segmentally iterated bulges (Fig. 4). The clefts elongated medially and met at the ventral midline, subdividing the bandlets into discrete ganglionic primordia. The fissure reached the midline within three segments (i.e. 6 blast cell clones) anterior to where

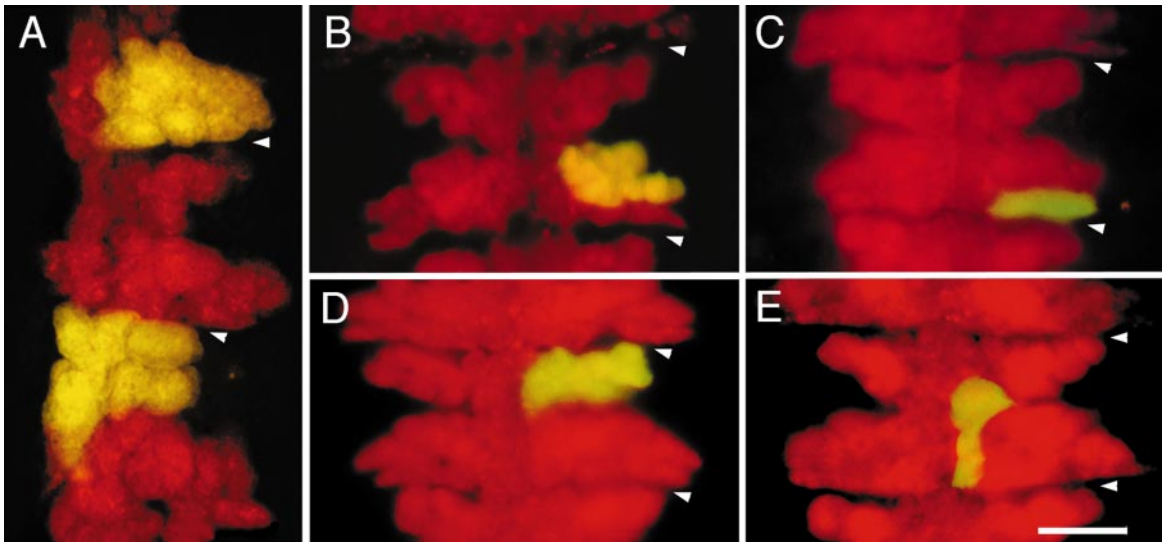


Fig. 5A–E Fissures form between nf.p and ns.a secondary blast cell clones. Double exposure photomicrographs, showing segments of embryos in which one (**A**) or both (**B–E**) N teloblasts were injected with RDA; later, primary (**A**) or secondary (**B–E**) n blast cells were injected with FDA and their doubly labeled clones therefore appear as *yellow*. *Arrowheads* identify the location of the fissures. **A** Confocal micrograph showing several planes of a preparation in which nf (*top*) and ns (*bottom*) primary blast cell fills were fixed at clonal ages ~50 and ~48 h, respectively. Secondary blast cell fills were fixed at clonal age ~55 h and photographed on a compound microscope: **B** nf.a; **C** nf.p; **D** ns.a; **E** ns.p (*Scale bar* 25 μm)

the cleft was first visible; since primary blast cells were born at the rate of 1.6 per hour, we conclude that the entire process of fissure formation is complete within ~4 h.

To investigate the possibility that the interganglionic fissures form between specific cells of the N lineage, we determined the location of each N-derived primary (nf and ns) and secondary (nf.a, nf.p, ns.a and ns.p) blast cell clone as the fissure formed (Fig. 5). For this purpose, we examined about 50 embryos each in which nf, ns, nf.a or ns.a clones were labeled and 5 embryos each in which nf.p or ns.p clones were labeled by direct injection of the primary or secondary blast cell (see Materials and methods). We found that the entire nf clone lay anterior to the fissure, and the ns clone lay posterior (Fig. 5A). There was no intermingling of nf- and ns-derived progeny at this stage (~50 h). The nf.p and ns.a clones contributed progeny that lined the anterior and posterior boundaries of the fissure, respectively; nf.p formed a narrow stripe of 4–6 cells at the posterior margin of one ganglionic primordium (Fig. 5C), while ns.a occupied the anterior lobe of the next posterior hemiganglion (Fig. 5D). The nf.a and ns.p clones contributed progeny internal to the boundaries of the fissure; nf.a-derived cells were contained within the posterior lobe of the hemiganglion (Fig. 5B), and ns.p occupied a medioposterior position that abutted the midline, connecting the ns.a subclone with nf (Fig. 5E). The relative positions of these clones within prospective ganglia indicate that nf.p- and ns.a-derived cells separate from each other, thus subdividing the initially continuous n bandlets into discrete ganglionic primordia.

As a result of fissure formation, the segmentally iterated bulges were separated into two distinct populations of cells in each segment (the anterior and posterior lobes, respectively). These lobes were oriented transverse to the anteroposterior axis of the germinal plate and marked the anterior and posterior margins of each prospective ganglion. The region between these projections is occupied by cells from other lineages, as described elsewhere (Weisblat and Shankland 1985; Torrence and Stuart 1986; Lans et al. 1993). As development proceeded, the nf clone expanded laterally further than the ns clone. Thus, on the basis of this criterion, the two clones could be distinguished from each other in the germinal plate (Weisblat et al. 1980a; Zackson 1984; Wedeen and Weisblat 1991; compare Figs. 1, 2 and 5).

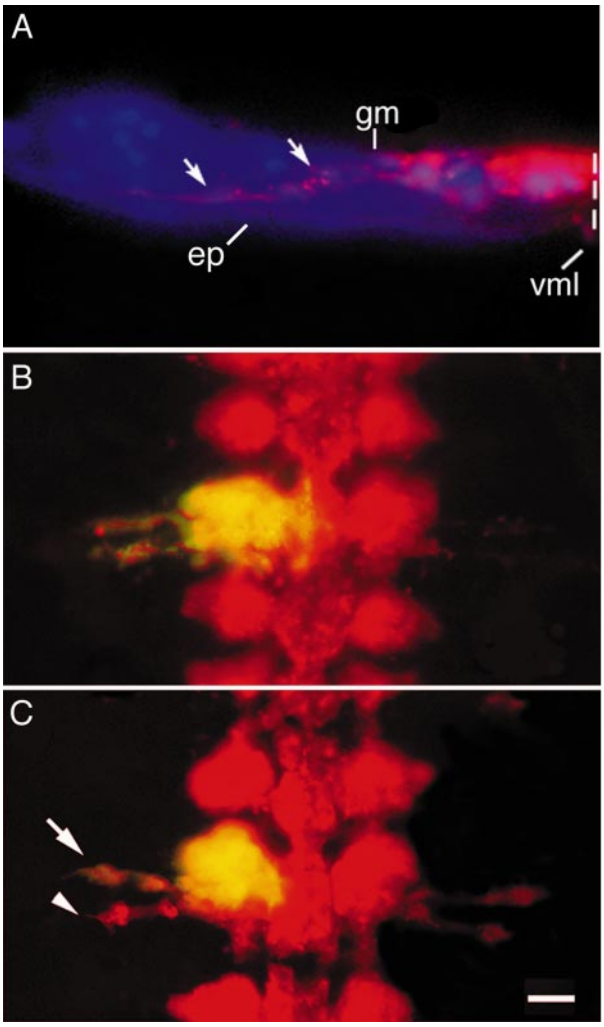


Fig. 6A–C Ventrolateral stripes of cells, arising from nf.a and nf.p clones, connect the ganglion to the ventral body wall. **A** Digitally merged photomicrographs showing one half of a germinal plate, sectioned (~10 μm thick) transverse to the longitudinal axis of the embryo at the level of an RDA-labeled nf clone (~65 h clonal age); this preparation was counterstained with Hoechst 33258 (*blue*). In this view, a ventrolateral stripe (*arrows*) extends from the dorsolateral margin of the ganglion (*gm*) toward the epidermis (*ep*) of the body wall. Ventral midline (*vml*) is at the *right*; dorsal is *up*. **B** and **C** Double exposure photomicrographs showing segmental ganglia from the dissected germinal plate of an embryo in which both N teloblasts were injected with RDA (*red*) and, later, individual nf or nf.a blast cells were injected with FDA; the doubly labeled clones appear *yellow* in these preparations. **B** In an nf clone fixed at ~65 h clonal age, both ventrolateral stripes are *yellow*, indicating that they arose from the doubly labeled nf clone. **C** In an nf.a clone fixed at ~65 h, the anterior stripe (*arrow*) is *yellow*, indicating that it arose from the nf.a clone, while the posterior stripe (*arrowhead*) is *red*, indicating that it arose from the nf.p clone (*Scale bar* 20 μm)

Ventrolateral stripes of nf-derived cells emerge from the posterior lobe of the ganglion: clonal ages 55–70 h

During this phase of development, the ns clone expanded along the anteroposterior axis, ultimately spanning the length of the ganglion. Most of this expansion was directed posteriorly along the medial portion of the nascent ganglion and resulted from clonal growth of the ns.p subclone, which increased to ~16 cells during this period. The nf clone, in contrast, expanded laterally, particularly along its ventral aspect, forming two stripes of cells near the ventral surface of the germinal plate. These nf-derived stripes were directly adjacent to each other with their cells forming a staggered mediolateral array. Examination of transverse sections at ~65 h clonal age revealed that these lateral stripes of cells transiently con-

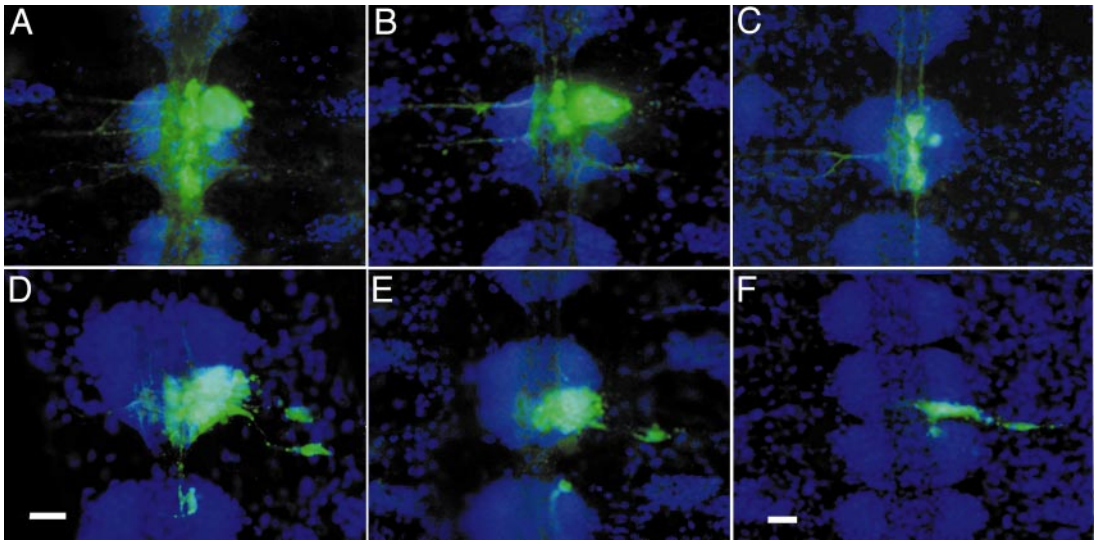


Fig. 7A–F Clonal contribution of definitive N-derived progeny. Digitally merged photomicrographs showing segmental ganglia containing the progeny of individual blast cells that were microinjected with FDA (*green*) and fixed at ~100 h clonal age. Germinal plates were counterstained with Hoechst 33258 to visualize the overall distribution of nuclei (*blue*). The injected blast cells in each panel are: **A** ns; **B** ns.a; **C** ns.p; **D** nf; **E** nf.a; **F** nf.p. Note that the cellular distributions shown in **B** and **C** sum to that shown in **A**, while those shown in **E** and **F** sum to that shown in **D**. In addition, while all ns-derived neurons are confined to within a single ganglion, the nf.a and nf.p clones each give rise to peripheral neurons and to cells in the adjacent posterior ganglion. In addition to the cell bodies, tracer-labeled axons are visible in one or more of the segmental or connective nerves in each panel (see Table 1 for details). (*Scale bar* 16 μm in **D**, 20 μm in all other panels)

nected the dorsal margin of the ganglion with the ventral body wall (Fig. 6A). No such projections were observed in the ns clone.

Previous work in *Helobdella triserialis* and *T. rude* (Ramirez et al. 1995) has shown that the leech *engrailed*-class gene is expressed in the more anterior of the two nf projections, and that the ns clone shows no detectable levels of the *engrailed*-class gene at this stage. Direct injection of secondary blast cells revealed that the nf.a clone gave rise to the anterior ventral stripe of cells; by subtraction we deduce that nf.p gives rise to the posterior ventral stripe of cells (Fig. 6B, C; see also Fig. 5B, C, in which the stripes of cells are just starting to form). Thus, cells expressing the leech *engrailed*-class gene arise from the nf.a subclone, and appear specifically in the anterior ventrolateral stripe (Fig. 6C). By clonal age ~75 h, most cells in both the nf.a and nf.p stripes had disappeared as in *Helobdella* (Wedeen and Weisblat 1991). Whether these cells die or migrate into the ganglion remains to be established.

The result that the transverse stripes of cells arise after the ganglionic primordia have already separated, and anterior to the fissures separating the primordia, suggests that, contrary to our previous hypothesis (Ramirez et al. 1995), these stripes are probably not the nf-derived cells required for separating adjacent ganglia.

Contributions of N-derived blast cell progeny to the mature ganglion: clonal age 100 h

To determine the relative positions of N-derived primary and secondary blast cell clones and their axonal projections in mature ganglia, blast cells were injected with FDA and fixed at ~100 h clonal age (Fig. 7). By this point, the embryos were at stage 11 of development and exhibited neuronally mediated behaviors including shortening and bending in response to tactile stimuli (Stent et al. 1992). The overall morphology of the nf and ns clones was similar to that observed at ~50 h clonal age (cf. Figs. 5, 7). In addition to an increase in cell number (Fig. 3), these clones also contained morphologically differentiated neurons, whose axons were visible in the segmental nerves and in the connectives of tracer-labeled specimens (Table 1).

Progeny derived from the ns clone were confined to a single ganglion (Fig. 7A); the ns.a subclone occupied the anterior lobe of each hemiganglion (Fig. 7B), and the ns.p subclone occupied a medio-lateral position that contacted ns.a at its anterior edge and extended to the posterior boundary of the ganglion (Fig. 7C). The ns.p clone also contributed at least one neuron lying lateral to the rest of the ns.p clone, and just posterior to ns.a (Fig. 7A, C). The nf clone contributed the majority of its progeny to the posterior lobe of the hemiganglion (Fig. 7D). The respective contributions of the nf.a and nf.p subclones are shown in Fig. 7E and F, respectively. The nf.a subclone occupied the anterior region of the posterior lobe, while nf.p remained as a relatively thin stripe of cells (compare with Fig. 5C) that formed the posterior boundary of the ganglion.

In contrast with the ns clone, several nf-derived neurons lay outside the ganglion, either peripherally (forming the ventrolateral stripes described above) or in the adjacent, posterior ganglion (Fig. 7D–F). Most of the cells in the ventrolateral stripes disappeared, but cells at the distal end of each stripe persisted and became the previously identified peripheral neurons of the N lineage (nz1, nz2 and nz3; Weisblat et al. 1978; Weisblat et al.

Table 1 Projections of N-derived neurons into connectives and segmental nerves. Embryos in which individual primary or secondary blast cells (top row) had been injected with FDA (clonal ages ~10-25 h) were fixed and dissected ~100 h later (i.e. clonal ages ranging from ~110 h for primary blast cell clones to ~125 h for secondary blast cell clones), then examined for the distribution of labeled axons within the connectives and segmental nerves. Only well labeled clones showing the normal distribution of labeled cell bodies were scored; the number of clones scored for each case is indicated in parentheses. Note that projections into the contralateral posterior connectives and into Faivre's nerve were seen from one or more of the secondary blast cell clones, but not from any of the primary blast cell clones from which they arose. This discrepancy may result from differences in clonal age at the time of fixation. Abbreviations not given in Figure 1 are AC, anterior connective; PC, posterior connective; AF, anterior Faivre's nerve; PF, posterior Faivre's nerve

	ns (3)	ns.a (5)	ns.p (2)	nf (5)	nf.a (6)	nf.p (2)
Ipsilateral						
AA	–	–	–	–	–	–
MA	–	–	–	–	–	–
PP	3	5	2	4	6	–
UP	1	5	–	5	–	2
AC	3	4	2	5	6	1
PC	3	4	2	5	6	1
Contralateral						
AA	3	5	–	–	–	–
MA	3	5	–	–	–	–
PP	3	–	2	5	5	–
UP	3	–	2	1	2	–
AC	2	3	2	1	1	–
PC	–	3	2	–	2	–
Midline						
AF	–	1	–	–	–	–
PF	–	–	1	–	–	–

1984; Torrence and Stuart 1986; Braun and Stent 1989a). The nz1 and nz2 neurons arose from the anterior, nf.a-derived stripe of cells (Fig. 7E); nz3 arose from the posterior, nf.p-derived stripe (Fig. 7F). This is consistent with the observations that, in *Helobdella*, the *engrailed*-class gene is expressed in nz1 and nz2 neurons (in addition to others) but not in nz3, a pattern which apparently persists throughout the life of the leech (Wedeen and Weisblat 1991; Lans et al. 1993). Cells originating from both nf.a and nf.p migrated into the adjacent, posterior ganglion and intermingled with cells derived from the posterior, ns.a clone (Fig. 7E, F). This migration began at ~90 h clonal age, well after the boundary between adjacent ganglia had been established (see Fig. 5). Finally, both ns and nf clones projected axonal processes into the segmental nerve tracts. Table 1 summarizes the main contributions of each primary and secondary blast cell clone to the connectives and segmental nerves.

Discussion

Formation of ganglionic primordia in leech

We have characterized the morphogenetic processes leading to gangliogenesis in the glossiphoniid leech CNS by following the clonal development of segmental founder cells (the nf and ns blast cells and their progeny) in the main neurogenic (N) lineage of *Theromyzon rude*. A key event in this process was the formation of a fissure between the nf.p and ns.a secondary blast cell clones over the course of 3–4 h (~50 h clonal age). This fissure subdivided an initially continuous column of cells (the n bandlets) into discrete ganglionic primordia. The mechanism by which this fissure forms remains to be determined. Possible mechanisms include: (1) active cell movements within the n bandlet leading to separation of the nf.p and ns.a clones; (2) cell movements in one or more of the adjacent lineages, such as the underlying mesodermal layer, that pull the nf.p and ns.a clones apart passively; and (3) clone-specific decreases in cellular affinities resulting in delamination of the nf.p and ns.a clones.

Previous experiments, in which nf and ns clones were deleted at the 2-cell stage (clonal age ~18–20 h in *T. rude*) are consistent with the notion of early differences between the nf and ns clones. In those experiments (Ramirez et al. 1995), ablation of an nf clone (which results in two ns clones becoming adjacent to each other in the germinal band) resulted in the fusion of the two hemiganglia on the affected side. By contrast, ablation of an ns clone (which results in two nf clones becoming adjacent to each other in the germinal band) reduced the size of the affected ganglion, but did not affect the separation of ganglia. One interpretation of this result is that, at some point after ablation of the nf clone, the two adjacent ns clones came together and then failed to separate. If so, this scenario may reflect differences between nf and ns clones in cell motility and/or affinity. We speculate that the nf.p clone serves as a "spacer" clone, of which the cells undergo a decrease in affinity with respect to the adjacent, ns.a-derived cells at ~50 h clonal age, allowing the ganglionic primordia to separate.

Significance of early *engrailed*-class gene expression in the N lineage

Previous studies have shown that the initial expression of the leech *engrailed*-class gene in the N lineage is in segmentally iterated stripes of nf-derived cells, prior to overt segmentation, and near the prospective morphological boundaries of future segments (Wedeen and Weisblat 1991; Lans et al. 1993; Ramirez et al. 1995). On this basis, and by analogy with what is known of the function of *engrailed* in *Drosophila* (Kornberg 1981a, b; DiNardo et al. 1985; Kornberg et al. 1985; Poole et al. 1985), it was proposed that one function of the annelid *engrailed* homologs may be in segmenting the nervous system by separating the n bandlets into discrete ganglionic primordia.

In the work reported here, we have extended the previous lineage analysis to show that the stripe of cells expressing the leech-*engrailed*-class gene arises more specifically from within the nf.a blast cell clone, and that a

posterior stripe of cells arises from the nf.p blast cell clone. We also determined that these stripes of cells appear at ~60 h clonal age, well after ganglionic primordia have separated. Moreover, the stripes of cells lie anterior to the ganglionic boundary. These data suggest that expression of the leech *engrailed*-class gene is not associated with the morphogenetic processes of ganglion separation.

Two observations suggest that the stripes of nf-derived cells may participate in forming segmental nerves that connect the ganglia to the body wall. First, as described here and previously (Braun and Stent 1989a; Wedeen and Weisblat 1991), identified peripheral neurons arise from the distal ends of both the anterior and posterior stripes of cells (nz1 and nz2 from the anterior, nf.a-derived stripe and nz3 from the posterior, nf.p-derived stripe). These neurons lie on the PP and UP branches of the posterior segmental nerve, respectively. Braun and Stent (1989b) also showed that nz3 is required for formation of the UP nerve. Second, we find that the stripes of cells project ventrolaterally from the dorsolateral aspect of the ganglion, forming a cellular bridge to the ventral body wall. These features make the two stripes of cells good candidates for providing a guidance pathway for the posterior nerve tracts. The expression of the leech *engrailed*-class gene may play a role in regulating the specificity of this process in the anterior stripe of nf.a-derived cells.

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